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Posttranslational processing and functional analysis of Tractin, an L1 family member in leech

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Posttranslational processing and functional analysis of Tractin, an L1 family member in Leech

by

Yingzhi Xu

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Yingzhi Xu

has met the dissertation requirements of Iowa State University

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For the Major Program

To my parents and sister, for their love, support and patience.

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GENERAL INTRODUCTION

Dissertation Organization

The dissertation presents the study of the posttranslational processing and functional analysis of Tractin. It begins with the background. The general features of neural cell adhesion molecules (CAMs) and immunoglobin superfamily (IgSF) are introduced. The structure and general function of the L1 CAM family are reviewed. Then the role of glycosylation and proteolytic processing is described. The overview of leech nervous system describes the advantage of using leech as a model. It is followed by a summary of previous findings of Tractin. The significance of this study is addressed.

After the general introduction, the dissertation is organized into two papers. The first paper was published in the *Journal of Biological Chemistry* in February of 2003. Yun Ji contributed to experiments of the biotinylation assay of Tractin in this paper. This paper describes the furin mediated proteolytic cleavage of the ectodomain of L1 CAM family member Tractin and how different fragments function in cell adhesion. The second paper was published in the *Journal of Neurobiology* in August of 1999. This paper describes the cloning of two glial cell specific intermediate filament proteins in leech, Macrolin and Gliarin.

The last part of the dissertation is the general conclusion. In this part the current knowledge about Tractin is discussed. A model based on the current work is presented and future work is proposed.

References cited in the general introduction and general conclusions are listed in the final chapter as Literature Cited.

Background

Neural cell adhesion molecules (CAMs)

During neurogenesis, precise and stereotypic patterns of connections are formed by specific navigation and correct pathway choices of neuronal growth cones in response to various particular combinations of guidance molecules (Tessier-Lavigne and Goodman, 1996; Chisholm and Tessier-Lavigne, 1999). Diffusible (netrin and semaphorin) and membrane attached molecules (semaphorin, ephrin, CAM, and ECM) act as attractive and repulsive signal cues. These signals interact with receptors on the growth cones and invoke different cellular responses to stabilize growth cone or promote growth cone collapse. In addition, neuronal growth cones express cell adhesion molecules (CAMs), which enable them to move by homophilic or heterophilic interaction with all the different environment cues.

The Ig superfamily (IgSF) of CAMs is characterized by the variability and complexity of their extracellular regions which contain multiple tandemly arranged domains: immunoglobulin (Ig)-like domains, fibronectin type III (FNIII)-like domains and other structure motifs (Johansen and Johansen, 1997). Ig and FNIII domains are protein-protein interaction modules. They can mediate Ca^{2+} independent cell-cell adhesion through homophilic or heterophilic binding at the cell surface. Some of the IgSF CAMs are attached to the membrane by glycosyl-phosphotidylinositol anchors whereas others have transmembrane and cytoplasmic domains interacting with signal transduction pathways and/or the cytoskeleton (Walsh and Doherty, 1997). Some neural CAMs have a large number of isoforms produced by alternative pre-mRNA (Hassel et al, 1997; Schmucker et al, 2000).

In addition, more diversity is generated by posttranslational modification such as glycosylation (Tang et al., 1994) and proteolytic processing (Johansen and Johansen, 1997).

The L1 subfamily of CAMs

L1 is the founder of the IgSF cell adhesion molecules. L1 family members are glycoproteins sharing common structures. They have six Ig-like domains, four to five FNIII domains, a transmembrane stretch and a conserved cytoplasmic domain of about 110 amino acids with an ankyrin binding motif and PDZ binding motif. The L1 family has four members in vertebrates: L1, neurofascin, CHL1 (a close homolog of L1), NrCAM, and three members in invertebrates: neuroglian in *Drosophila*, Tractin in leech, and LAD-1 in nematode (Brümmendorf et al., 1998; Chen et al., 2001). L1 CAMs are primarily expressed in the nervous system and play a crucial role in multiple processes in the development of the nervous system including neuronal migration, myelination, neurite outgrowth, growth cone morphology, axon fasciculation, and hippocampal long term potentiation (Hortsch, 1996; Brümmendorf et al., 1998).

Mutations in the human L1 gene on the X chromosome result in severe neurological disorders termed as CRASH syndromes (for corpus callosum hypolasia, mental retardation, adducted thumbs, spastic paraplegia, X-linked hydrocephalus; Fransen et al., 1998). L1 knockout mice show severe brain abnormalities similar to those observed in human (Dahme et al., 1997; Cohen et al., 1998; Rolf et al., 2001). The severity of the mutant phenotype is highly dependent on the genetic background suggesting the participation of modifier genes (Dahme et al., 1997; Rolf et al., 2001).

The extracellular Ig and FNIII domains mediate the homophilic binding of L1 CAMs in *trans* and also heterophilic *cis* or *trans* binding with other molecules, including RGD

specific integrin, ECM molecules, proteoglycan-type molecules and other IgSFs, such as NCAM, F3/F11 and axonin/TAG-1 (Brümmendorf et al., 1998; Hortsch, 2000). Many human pathological missense mutations in L1 affect its homophilic and heterophilic binding activities (Angelis et al., 1999). The cell adhesion mediated by homo- or heterophilic interaction plays an important role in neurite outgrowth and axon fasciculation (Lemmon et al., 1989; Zhao and Siu, 1995). In addition, L1 might function as a long a range repulsive cue in axon guidance by serving as a co-receptor with neuropilin for Sema3A-mediated signal transduction (Castellani et al., 2000).

The crystal structure of hemolin shed some light on how L1 CAMs interact homophilically and/or heterophilically with other IgSF molecules (Su et al., 1998). Hemolin is a member of the IgSF sharing high homology with the first four Ig domains of the L1CAMs. The four Ig domains of hemolin form a horseshoe-shaped structure. A very similar horseshoe conformation was found in the crystal structure of four Ig domains D1-D4 of axonin-1 (Freigang et al., 2000). The same structure and its open form are also observed in L1 (Schürman et al., 2001). The sharp bending between D2 and D3 Ig domains establishes a close contact between D1 and D4 and between D2 and D3 in the horseshoe shaped closed monomer. The open monomer can form domain-swapped dimers or multimers with intramolecule D1-D4 and D2-D3 pairs to mediate cell-cell adhesion. Neurocan is a chondroitin sulfate proteoglycan. It has been reported that neurocan can block L1 mediated homophilic interaction by binding to its first Ig domain in open form thus prevents the trans D1-D4 pair formation (Olezewski et al., 2000) (as shown in figure 1).

L1CAM-mediated cell adhesion is stabilized through the interaction with ankyrin, which links L1CAMs to the spectrin-actin cytoskeleton. L1 and neuroglian can interact with cytoskeletal ankyrin and recruit ankyrin to the cell-cell contact sites by L1-mediated cell adhesion (Dubreuil et al., 1996; Hortsch et al., 1998b). This interaction is important for the

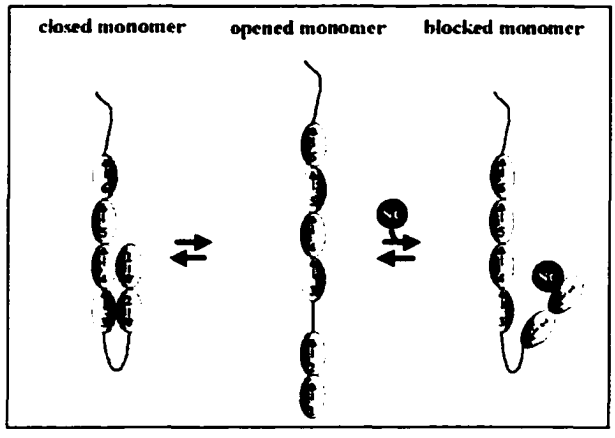

Fig. 1 Diagram of the horseshoe shape structure of L1.

The horseshoe-like structure of the six Ig domains of L1 based on the crystal structure of hemolin is shown on the left. Transient formation of linear open form is shown as an equilibrium in the middle part of the figure. Neurocan binding to the first domain of the L1 open form removes it from the equilibrium and is shown on the right side. Binding of neurocan prevents flipping back into the horseshoe form but also prevents homophilic interaction with open L1 proteins on another cell (Adapted from Olezewski et al., 2000).

stability of ankyrin (Bouley et al., 2000) and may be involved in the generation and maintenance of specialized membrane subdomains such as axon initial segments and the nodes of Ranvier in vertebrates (Bennett and Chen, 2001). The neuroglian and ankyrin interaction is independent of the extracellular domain sequence (Hortsch et al., 1998a). The residues critical for this interaction are localized in the highly conserved 26-residue region in the cytoplasmic domain, which has a random coil structure (Hortsch, 2000; Garver et al., 1997). In this region residue FIGQY are completely conserved in all L1 family members. Phosphorylation of the tyrosine within the FIGQY sequence in response to FGF and NGF completely abolished the association between ankyrin and neurofascin and reduced the neurofascin mediated cell adhesion (Tuvia et al., 1997; Garver et al., 1997). One missense mutation results in conversion of tyrosine of FIGQY to histidine (Y1229H) and clinical disease (Kendrick and Doherty, 1998). The Y1229H mutation abolishes both ankyrin binding (Needham et al., 2001) and tyrosine signaling.

Homophilic L1-L1 *trans* binding might lead to the *cis* heterodimerization of L1 with fibroblast growth factor (FGF) receptor through their extracellular domains. This interaction leads to activation of the tyrosine kinase domain of the FGFR to phosphorylate tyrosine in the FIGQY sequence (Denwrick and Doherty, 1998). It has been shown that EGF and FGF receptors are downstream targets of neuroglian activity (Garvia-Alonso et al., 2000). In *C. elegans* LAD-1 is a substrate of the FGFR pathway and the phosphorylation of FIGQY occurs downstream of the FGFR-activated Ras pathway (Chen et al., 2001). FIGQY phosphorylated L1 CAMs are located at migrating neurons and specialized cell junctions (Chen et al., 2000; Jenkins et al., 2000). In addition, phospho-FIGQY L1 CAMs are confined to the ankyrin free regions (Jenkins, et al., 2000). Thus phosphorylation of the FIGQY sequence in L1 CAMs defines ankyrin-containing and ankyrin-free domains to regulate the ankyrin-dependent adhesion and ankyrin-independent intracellular signal transduction.

The phospho-FIGQY neurofascin can specifically associate with doublecortin in the migrating neurons (Kizhatil et al., 2002). Doublecortin is a microtubule-associated protein mutated in X-linked lissencephaly (XLIS), a neuronal migrating disorder (Gleeson et al., 1998; Francis et al., 1999). Doublecortin also interacts with LIS1, another gene of XLIS, which plays a critical role in nucleokinesis in *Aspergillus* (Feng and Walsh, 2001). All these suggested that doublecortin might link neurofascin to microtubule functioning in neuronal migration directly or through LIS1.

In addition to the ankyrin-binding motif, the XS/TXV PDZ binding motif has been found at the COOH-terminal of all L1 CAMs. PDZ (PSD-95/SAP50/ZO-1) domains are multifunctional protein-binding modules. Despite the diverse sequences comprising the PDZ domain, the secondary structures of different PDZ domains are highly conserved, containing a core of six β-sheets and two α-helices (Doyle et al., 1996). PDZ proteins act as adaptors and attach to the C termini of membrane receptors (Torres et al., 1998) and ion channels (Kim et al., 1995) via some of their PDZ domains, while also binding to signaling proteins (Vries et al., 1998) via other PDZ domains or other modules. The PDZ domain binding is selective and the specificity is determined by the -2 position of the bound peptide. The only PDZ domain containing interaction partner found for L1 CAMs is syntenin-1, which is the receptor of syndecans (Groothans et al., 2000). Syntenin-1 was identified as a neurofascin-binding protein in yeast two hybrid screening (Koroll et al., 2001). It also interacts with other neural receptors. Syntenin-1 can form homodimer or heterodimer with syntenin-2 through its PDZ domain. Therefore neurofascin might function in a complex network acting as a cross-talking receptor through syntenin with its multiple interacting partners (Brümmendorf, 2001).

All known L1 CAMs in vertebrates contain a neuronal specific sequence RSLE from alternatively splicing (Hortsch, 2000). Together with a proceeding tyrosine, the sequence YRSLE comprises an internalization signal in AP-2 mediated endocytosis (Kamiguchi et al.,

1998). Phosphorylation of Y1176 prevents L1 binding to AP-2, an adaptor required for clathrin-mediated internalization, thus inhibiting the internalization of L1 (Schafer et al., 2002). The internalization of L1 is required for the activation of the MAPK signal cascade, which regulates L1 intracellular trafficking (Schafer et al., 1999).

Glycosylation

Differential glycosylation plays an important role in protein function regulation. The diversity of cell surface carbohydrates provides means for cell specific recognition (Lis and Sharon, 1993; Dwek, 1995). In the nervous system distinct carbohydrate epitopes such as that recognized by anti-HRP antibody in insects and HNK-1/L2 in vertebrates are widely expressed on glycoproteins (Jessell et al., 1990). One example of how regulated glycosylation affects neural pathway formation is the modulation of polysialic acid (PSA) on NCAM. PSA regulates axon guidance and targeting, cell translocation and migration during development (Rutishauser, 1996). PSA on NCAM negatively regulates cell-cell adhesion. In the chicken limb bud the up-regulation of PSA allows the axons to defasciculate into their proper pathway (Tang et al., 1994). In addition the interaction between L1 and NCAM is mediated by oligomannosidic glycans and the interaction stimulates neurite outgrowth (Hoestkorte et al., 1993). In the vertebrate olfactory system, stage- and position specific carbohydrate serves as a marker in sorting and bundling the axons into specific tracts and establishing the topography of connections between olfactory neuroepithelium and the olfactory bulb (Key and Akeson, 1991; Dowsing et al., 1996). Therefore glycosylation plays an important role in neural pathfinding, axon fasciculation and defasciculation during development.

Posttranslational processing

The secreted forms of both type I and type II integral membrane proteins including CAMs, growth factor and cytokine receptors, and receptor ligands are derived from selective posttranslational proteolysis (Nakayama, 1997). In some cases it may be a process for rapid down-regulating the protein from the surface, such as the proteolysis of APP (Lammich et al., 1999). In others it may generate a soluble form of the protein that has functional properties different from those of the membrane-bound form (Hooper, et al., 1997). Furin is a calcium dependent proprotein convertase involved in processing various precursor proteins. The consensus site for furin cleavage is RxR/KR↓. Arg residues at the P1 and P4 positions are essential for the cleavage, whereas the P2 basic residue is not, but it greatly enhances the processing efficiency (Thomas, 2002). For example, in order to generate a functional Notch receptor in *Drosophila* the protein is cleaved constitutively by a furin convertase (Logeat et al., 1998) to form a disulfide linked heterodimer (Blaumueller et al., 1997). Furin also plays an essential role in activating the metalloproteases (Thomas, 2002).

Metalloproteases are zinc-dependent proteolytic enzymes that include the matrix metalloprotease (MMP) and disintegrin metalloprotease (ADAM) families (Chang and Werb, 2000). ADAMs are transmembrane proteins that contain disintegrin and metalloprotease domains, indicative of cell adhesion and protease activity. Loss-of-function mutations in the *kuzbanian* gene, which codes for a disintegrin metalloprotease in the *Drosophila* embryo show that its proteolytic activity is required for axonal extension (Fambrough, et al., 1996). Netrin mediated chemoattraction is regulated by DCC. Blocking of cleavage of DCC by metalloprotease inhibitor potentiates axon outgrowth (Galko and Tessier-Lavigne, 2000). Contact-mediated repulsion via an Eph receptor and ephrin A2 requires the cleavage of ephrin-A2 by Kuz, which allows efficient axon detachment and withdrawal (Hattori, et al.,

2000). ADAM10 mediated L1 ectodomain shedding at the membrane-proximal site can promote cell migration by autocrine binding to integrins (Gutwein et al., 2000; Mechtersheimer et al., 2001). All these suggest that metalloprotease mediated processing of neural CAM might be a general theme in axon outgrowth, fasciculation and defasciculation, and cell migration.

Overview of leech nervous system

Leech is an excellent model in which to study the complex mechanism of neuronal development because of its relatively simple and segmentally iterated nervous system and easy accessibility for functional perturbation experiments (Muller et al., 1981; Jellies and Kristan, 1988; Zipser et al., 1989). The development of both central and peripheral nervous system in leech proceeds in rostro-caudal sequence (Stent et al., 1982). Each posterior segment is approximately 2.5 h later than the more anterior one (Jellies and Kristan, 1991). Thus the leech embryo exhibits segments in different stages spanning over 2-3 days, which greatly facilitated the analysis of axonal outgrowth (Johansen et al., 1992).

The leech central nervous system consists of a head brain with 4 fused ganglia, a tail brain with 7 fused ganglia and 21 iterated segmental body ganglia linked to each other by connectives (Muller et al., 1981). Each of the segmental ganglia contains about 400 neurons, which are relatively large, some being up to 100 μm in diameter (Macagno, 1980). In each hemisegment there are seven peripheral sensory sensillars, which are clusters of mixed sensory neurons composed of chemoreceptors, photoreceptors and mechanoreceptors found on the central annulus of each segment. The sensory sensillar neurons in each hemisegment send their axons towards CNS through different nerve roots (Muller et al., 1981; Johansen et al., 1992). Axons of sensillar neurons project in tightly fasciculated bundles through the

periphery into the central nervous system where they bifurcate and segregate into four well-defined and stereotypically located fascicles in each of the central connectives (Johansen et al., 1992; Briggs et al., 1995; Jellies et al., 1995). The stereotyped peripheral nerve formation is guided by CNS-derived guidance cues (Jellies et al., 2000). In CNS ablation experiments the axons of the peripheral neurons form local fasciculated tangles instead of orderly projections and fail to reach the CNS (Jellies et al., 1995). Ectopically transplanted CNS ganglia are necessary and sufficient to guide the peripheral sensory axons towards the CNS when in physical contact. Thus the stereotyped peripheral nerve formation is guided by CNS-derived cues (Jellies et al., 2000).

The sensory neurons and fascicles display various glycoepitopes identified by different monoclonal antibodies (Lan3-2, Lan4-2, Lan2-3, Laz7-79, Laz2-369, Laz6-212) (McKay et al., 1983; Peinado et al., 1987; Zipser et al., 1994). These glycoepitopes have different expression patterns in both PNS and CNS. The Lan3-2 epitope is expressed on all sensory neurons and nerve tracts. Some other epitopes such as Laz2-369, Lan2-3, Laz7-79, are expressed on subsets of the sensory nerve tracts. These epitopes also show different temporal expression patterns in development. While the Lan3-2 epitope is detected from the earliest onset of growth cone extension, the Laz2-369 epitope is not expressed on axons before they have reached and bifurcated in the CNS and with a 12-24 h delay compared to Lan3-2. The temporal expression differences in glycoepitopes suggest developmental regulation of interactions within the CNS (Jellies et al., 1994; 1995; 1996). All this compelling evidence indicates that a hierarchy of distinct carbohydrate structures expressed in neurons are involved in regulating neuronal pathway formation in the leech nervous system.

In addition to neurons, glial cells, by number and volume, make up a large fraction of central nervous system tissue in leech. The communication between glia and neuron plays an

important role in nervous system development. In addition to microglial cells, there are four types of macroglial cells within the central nervous system in each leech segment: (a) one pair of macroglia cells located in the connectives wrap all axons traveling in the two lateral connectives and Faivre's nerve, (b) six packet glial cells envelope the neuronal cell bodies in each ganglion, (c) two large glial cells surround axons and neuronal processes in the neuropil, (d) two glial cells ensheathe axons in the peripheral nerve roots (Flaster and Zipser, 1987). In leech there are two glial cell-specific intermediate filaments. Gliarin is expressed in all glial cells in the nervous system, whereas Macrolin is expressed only in the giant macroglia cell enveloping the axon in the ganglionic connective. In addition filarin is an intermediate filament expressed in all neuron cells. The cytoplasmic IF GFAP serves as a marker for astrocytes in vertebrates (Fuchs and Weber, 1994). As the only two glial cell-specific intermediate filaments found thus far in invertebrates, Gliarin and Macrolin will be good markers for analyzing glia-axon interactions in nerve formation.

Tractin, an L1 family member in leech

Tractin, one of the antigens recognized by the Lan3-2 antibody, is a member of the L1 family. It contains 6 Ig-like domains, 4 FNIII-like domains, an acidic domain, 12 repeats of a proline- and glycine-rich motif (PG/YG) with sequences similar to type IV collagen, a transmembrane domain and an intracellular tail with ankyrin and PDZ binding motifs (Huang et al., 1997). Tractin is constitutively processed at two cleavage sites, one in the third FNIII domain and the other proximal to the transmembrane domain (Huang et al., 1997; Jie et al., 2000 (as shown in Fig 2). Yeast two-hybrid interaction assays reveal that Tractin can interact with ankyrin through the cytoplasmic ankyrin-binding motif (Jie et al., 2000).

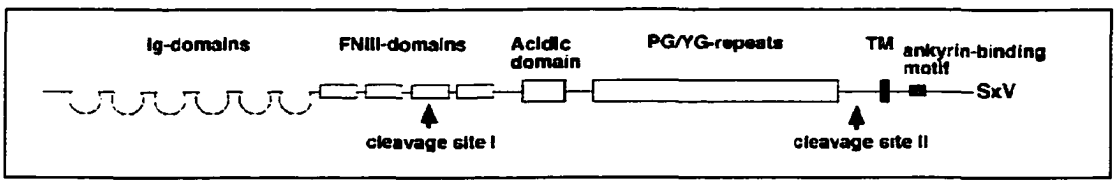

Fig. 2 Diagram of the structure of Tractin.

An unusual feature of Tractin is the presence of the acidic region and the PG/YG repeats which are similar to Type IV collagen. Type IV collagen is a major component of the extracellular matrix. Collagenase digestion experiments provide strong evidence that PG/YG repeats are collagenous (Jie et al., 2000). The COOH-terminal fragment of Tractin might form an integral transmembrane collagen which is linked to the cytoskeleton through ankyrin binding or other PDZ molecules. Thus Tractin may function as an extracellular matrix component to provide a framework for axon outgrowth and axon fasciculation.

The NH_2-terminal secreted fragment of Tractin is highly glycosylated. The core protein of Tractin is expressed by both peripheral and central neurons but is differentially glycosylated with the Lan3-2, Lan4-2 and Laz2-369 glycoepitopes only in sets or subsets of peripheral sensory neurons that form distinct fascicles in the CNS (Huang et al., 1997; Jie et al., 2000). These different glycoepitopes show a temporal modification pattern (Jie et al., 2000) and function differentially in neurite outgrowth and synaptogenesis. Perturbation of Lan3-2 antibody leads to an inhibition of neurite extension, truncated fascicle formation and a decrease in synaptogenesis (Zipser et al., 1989; Huang et al., 1997; Tai and Zipser, 1998). In contrast, perturbation with Laz2-369 antibody leads to enhanced neurite and filopodial sprouting as well as an increase in synapse formation (Song and Zipser, 1995; Tai and Zipser, 1999).

Significance of the study

CAMs mediate axon guidance and pathway formation through a complex hierarchy of molecular interactions and signal transduction events. Thus it is important to identify the molecules interacting in these processes and how these molecules function in axon outgrowth, axon guidance and axon fasciculation in nervous system development. Mutations in L1 cause severe brain abnormalities and mental retardation in humans. It is still not clear how these mutations lead to brain defects. The leech nervous system is relatively simple and well defined and easily accessed by antibody perturbation. The study of Tractin will provide valuable information in understanding the functions of L1 in nervous system development. Furthermore the study will also help elucidate the role of glycomodification in selective axon outgrowth and fasciculation. Therefore the work in the dissertation provides new insight into the mechanism of neural connection and brain development mediated by L1 family members.

PROTEOLYTIC CLEAVAGE OF THE ECTODOMAIN OF THE L1 CAM-FAMILY MEMBER TRACTIN

A paper published Journal of Biological Chemistry

Ying-zhi Xu, Yun Ji, Birgit Zipser[+], John Jellies[†],
Kristen M. Johansen and Jørgen Johansen*

SUMMARY

Tractin is a member of the L1-family of cell adhesion molecules in leech. Immunoblot analysis suggests that Tractin is constitutively cleaved *in vivo* at a proteolytic site with the sequence RKRRSR. This sequence conforms to the consensus sequence for cleavage by members of the furin family of convertases and this proteolytic site is shared by a majority of other L1-family members. We provide evidence by furin specific inhibitor experiments, by site-specific mutagenesis of Tractin constructs expressed in S2 cells, as well as by Tractin expression in furin deficient LoVo cells that a furin convertase is the likely protease mediating this processing. Cross-immunoprecipitations with Tractin domain specific antibodies suggest that the resulting NH_2- and COOH-terminal cleavage fragments interact with each other and that this interaction provides a means for the NH_2-terminal fragment to be tethered to the membrane. Furthermore, in S2 cell aggregation assays we show that the NH_2-terminal fragment is necessary for homophilic adhesion and that cells

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expressing only the transmembrane COOH-terminal fragment are non-adhesive. However, tethering of exogeneously provided Tractin NH_2-terminal fragment to S2 cells expressing only the COOH-terminal fragment can functionally restore the adhesive properties of Tractin. This novel protein-protein interaction mechanism may be of general biological relevance for L1 family CAMs processed at this proteolytic site.

INTRODUCTION

The L1 family of cell adhesion molecules (CAMs)[1] is constituted of transmembrane proteins with six Ig-like domains and four to five FNIII-like domains in the extracellular domain (1). In addition, they are characterized by a highly conserved cytoplasmic segment that contains an ankyrin binding site. The L1 family currently has four members in vertebrates (L1, NrCAM, CHL1, and neurofascin) as well as three invertebrate members, neuroglian in arthropods, Tractin in leech, and LAD-1 in nematodes (1, 2). Although also expressed in other tissues L1 family CAMs are predominantly found in the nervous system where they have been implicated in a number of different cellular processes such as neuronal cell migration, myelination, axonal growth, axon fasciculation, and axon guidance (3). Of particular interest is that mutations in the human L1 gene on the X chromosome result in a complex human mental retardation syndrome referred to as CRASH (4, 5) the symptoms of which include hydrocephalus, spastic paraplegia, and corpus callosum agenesis (6). Furthermore, L1 knockout mice have been generated which show severe brain abnormalities similar to those observed in humans (7, 8). However, the actual developmental mechanisms by which mutations in the L1 gene causes these brain defects are not well understood. In humans and mice the severity of the phenotypes observed is highly dependent on the genetic background suggesting the participation of modifier genes (8). Thus, the neurological

phenotypes of L1 mutations observed in mammals as well as in *Drosophila* (9) cannot be accounted for by disruption of L1's adhesive function alone, but is likely to also involve interactions with extracellular ligands and intracellular signaling pathways linked to cytoskeletal elements (3, 10). For example, L1 family CAMs have an array of different protein domains that in addition to participating in homophilic interactions also function in heterophilic interactions with other CAMs, integrins, and extracellular matrix proteins (1, 3).

The diversity in the structure and potential functional repertoire of neural CAMs is further amplified with the existence of many splice variants and various posttranslational modifications such as differential glycosylation and proteolytic processing (11-13). There is a growing body of evidence that secreted forms of both type I and type II integral membrane proteins including CAMs, growth factor and cytokine receptors, and receptor ligands are derived from selective posttranslational proteolysis (14). The biological function of the proteolytic cleavage of transmembrane proteins is still not well characterized and may vary. In some cases it may be a process for rapidly down-regulating the protein from the cell surface, in others it may be to generate a soluble form of the protein that has functional properties different from those of the membrane bound form (12). In some cases the processing may be necessary for biological activity. For example, in order to generate a functional Notch receptor in *Drosophila* the protein is cleaved constitutively by a furin convertase (15) to form a disulfide linked heterodimer (16, 17). Furthermore, loss-of-function mutations in the *kuzbanian* gene which codes for a disintegrin metalloprotease in *Drosophila* embryos show that its proteolytic activity is required for axonal extension (18). Recently, it has been demonstrated that ectodomain shedding of human and mouse L1 can promote cell migration by binding of the secreted ectodomain to integrins (19).

In this study we characterize the proteolytic processing of the invertebrate L1 family member Tractin in the S2 cell line. We provide evidence that the ectodomain of Tractin is

constitutively cleaved by a furin-like convertase at a dibasic cleavage site conserved in many L1 family member CAMs. We further demonstrate that the shedded ectodomain can be tethered to the membrane by binding to the cleaved transmembrane fragment and that this interaction promotes homophilic adhesion. We propose that this novel mechanism may be a general feature for tethering the NH_2-terminal domain of L1-family CAMs generated by cleavage at this site to the membrane.

MATERIALS AND METHODS

Experimental preparations

For the present experiments we used the two hirudinid leech species *Hirudo medicinalis* and *Haemopis marmorata*. The leeches were either captured in the wild or purchased from commercial sources. Dissections of nervous tissue were performed in leech saline solutions with the following composition (in mM): 110 NaCl, 4 KCl, 2 $CaCl_2$, 10 glucose, 10 HEPES, pH. 7.4.

Antibodies

The previously reported Tractin monoclonal antibodies (mAbs), Laz6-56, 4G5, 3A11, and 3A12 (20-22) were used in these studies. In addition a new antibody, 1H4, was made to a glutathione-S-transferase (GST)-fusion protein of the PGYG-domain of Tractin in the pGEX vector system (Pharmacia) encompassing the sequence T1184-P1662. The correct orientation and frame of the construct was verified by sequencing the insert. The fusion protein was expressed in XL1-Blue cells (Stratagene), harvested, and purified over glutathione agarose (Sigma) columns according to standard protocols (Pharmacia). Balb C mice were injected with 50 μg of the purified fusion proteins at 21 day intervals. After the

third boost spleen cells of the mice were fused with Sp2 myeloma cells and monospecific hybridoma lines established using standard procedures (23). All procedures for monoclonal antibody production were performed by the Iowa State University Hybridoma Facility. V5- and Fc-antibody were obtained from commercial sources (Invitrogen and ICN, respectively).

Full length and Tractin deletion constructs

A full length Tractin construct (M1-V1880) was cloned into the pMT/V5-HisB vector (Invitrogen) using standard procedures (24). A series of deletions of this construct were made in the pMT/V5-HisB vector: ΔAc with the acidic domain deleted from P1089-P1161, ΔPGYG with the PGYG domain deleted from G1197-L1656, and ΔAc/PGYG with both the acidic and the PGYG domains deleted from P1089-L1656. All these constructs contain an in frame V5 tag at the COOH-terminal end. In addition, a series of N-terminal sequence deletion constructs were cloned into the pMT/BiP/V5-HisB vector (Invitrogen) which contains the *Drosophila* BiP secretion signal to ensure the proper processing of the expressed proteins in transfected S2 cells. The CTF construct contained all the sequence (S905-V1880) after the 1st cleavage site, the ΔIg/FN1-3 construct contained the sequences (P957-V1880) after the 3rd FNIII domain, and the ΔIg/FN construct contained the remaining sequences (P1092-V1880) after the 4th FNIII domain. Full length Tractin (M1-V1880) was also cloned into the pcDNA3.1 vector (Invitrogen) for mammalian cell transfections in LoVo (ATCC) and HEK293T (Genehunter) cells. For expression in COS cells (ATCC) the NH_2-terminal fragment of Tractin from M1-K890 was ligated in frame to a human IgG Fc fragment (AF150959) in the pcDNA3.1 vector (Invitrogen) resulting in the NTF-Fc fusion construct. For control experiments the Fc fragment alone was also cloned into the pcDNA3.1 vector. For SDS-PAGE mobility comparisons a GST-fusion protein containing the sequences of the acidic and the PGYG-domain (E1093-T1660) was made and expressed in the pGEX vector

system (Pharmacia) as described above. In addition, a full-length Tractin construct where the sequence RKRRSR was changed into AAAASA was generated by PCR mutagenesis using standard procedures (24). The fidelity of all constructs were verified by sequencing at the Iowa State University Sequencing Facility.

Expression of Tractin constructs in transfected cells

Drosophila Schneider 2 (S2) cells were grown in Shields and Sang M3 insect medium (Sigma) containing 10% fetal bovine serum (FBS) and antibiotics. S2 cells were transfected with Tractin cDNA clones using a calcium phosphate transfection kit (Invitrogen). Stable lines of each Tractin subclone were established by co-transfection with pCoHYGRO (Invitrogen) to confer hygromycin resistance. The stable lines were maintained with 300 μg/ml hygromycin (Invitrogen) in the culture medium. The expression of Tractin subclones was induced with 0.5 mM $CuSO_4$. COS cells and HEK293T cells were grown in DMEM medium with 10% FBS. LoVo cells were grown in Ham's F12 medium with 10% FBS. COS, HEK293T, and LoVo cells were plated overnight to reach 80% confluence and transiently transfected with Tractin/pcDNA3.1, NTF-Fc/pcDNA3.1, or Fc/pcDNA3.1 using lipofectamine as per the manufacturer's instructions (Invitrogen). Cells expressing Tractin or modified versions of Tractin were harvested 24-48 h after transfection and pelleted by centrifugation at 2,000 rpm for 5 min. For some experiments the supernatant was collected for further analysis by SDS-PAGE and immunoblotting. The pellet was resuspended in cell lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, pH 7.4) at 37°C for 10 min before centrifugation at 12,000 rpm for 10 min. In some cases the samples were additionally sonicated. The resulting supernatant was collected and analyzed with SDS-PAGE and immunoblotting. NTF-Fc and Fc protein for cell aggregation assays in cultured S2 cells was obtained by harvesting NTF-Fc or Fc expressing COS cells 48 h after transfection. The cells

were resuspended and sonicated in S2 cell culture medium at $5x10^6$ cells/ml. The supernatant was collected after centrifugation at 12,000 rpm for 10 min and applied to cultured S2 cells expressing various Tractin deletion constructs for cell aggregation analysis. The levels of NTF-Fc and Fc protein in the lysate was verified by SDS-PAGE and immunoblotting.

Biochemical analysis

SDS-PAGE and immunoblotting. SDS-PAGE was performed according to standard procedures (25). Electroblot transfer was performed as in Towbin et al. (26) with 1X buffer containing 20% methanol and in most cases including 0.04% SDS. For these experiments we used the Bio-Rad Mini PROTEAN II system, electroblotting to 0.2 μm nitrocellulose, and using anti-mouse HRP-conjugated secondary antibody (Bio-Rad) (1:3000) for visualization of primary antibody diluted 1:1000 in Blotto. The signal was developed with DAB (0.1 mg/ml) and H_2O_2 (0.03%) and enhanced with 0.008% $NiCl_2$ or visualized using chemiluminescent detection methods (ECL kit, Amersham). The immunoblots were digitized using an Arcus II scanner (AGFA).

Immunoprecipitation. Immunoprecipitations were performed at 4°C. Dissected *Haemopis* leech nerve cord were homogenized in extraction buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 0.2% NP-40, 0.2% Triton X-100, pH 7.4 containing the protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and Aprotinin from Sigma and the homogenate (20 μl) incubated with the nonspecific mouse IgG conjugated to protein G beads for 2 h. The resulting supernatant was then incubated with anti-Tractin antibody conjugated to protein G beads (10 μl) overnight. For immunoprecipitations from Tractin construct expressing S2 cell lines the cells were harvested and sonicated in immunoprecipitation buffer (20 mM Tris-HCl, 10 mM EDTA, 1 mM EGTA, 150 mM NaCl,

0.2% Triton X-100, 0.2% NP-40, pH 7.4) containing the protease inhibitors PMSF and Aprotinin. Appropriate amounts of antibody were conjugated to 10 µl protein G sepharose beads (Sigma) for 2 h on ice. In the case of immunoprecipitations from S2 cell culture medium the cell medium was incubated with antibody conjugated protein G sepharose beads overnight. After a brief spin for 20 s at 2000 rpm the supernatant was discarded and the immunoaffinity matrix resuspended and washed 3 times with 400 µl of extraction buffer for 15 min. The final pellet was resuspended in 20 µl of SDS-PAGE sample buffer and boiled for 5 min before centrifugation and analysis of the supernatant by SDS-PAGE and immunoblotting.

For co-immunoprecipitation experiments with Tractin deletion constructs Laz6-56 antibody was conjugated to protein G sepharose beads as described above. The resulting Laz6-56 conjugated immunobeads were then incubated for 12 h with 400 µl of culture medium obtained from S2 cells that had expressed full length Tractin for 12 h. After a brief spin for 20 sec at 2000 rpm the supernatant was discarded and the immunobeads resuspended and washed 3 times with immunoprecipitation buffer. The immunobeads were then incubated with 200 µl of lysate from ΔIgFN, ΔIgFN1-3, and CTF construct expressing S2 cells for 12 h. The resulting immunoprecipitate was processed and analyzed by SDS-PAGE and immunoblotting as described above.

Biotinylation assays. S2 cells transfected with the Tractin/pMT-V5 construct were induced with 0.5 M $CuSO_4$ for 24 h and subsequently washed three times with ice-cold PBS (pH 8.0). Cells were resuspended at a concentration of $2.5x10^7$ cells/ml in PBS (pH 8.0). Sulfo-NHS-LS-Biotin (Pierce) was added to cells at 1 mg/ml or mock treated for 30 min at room temperature. The cells were then washed three times with ice-cold PBS to remove any remaining biotinylation reagent. The cells were sonicated in immunoprecipitation buffer as described above at a concentration of $5x10^7$ cells/ml. The extracts were precleared with

protein G beads at 4°C for 2 h and the precleared extracts were directly immunoprecipitated with 1H4 antibody conjugated to protein G Sepharose matrix or incubated with streptavidin-agarose beads overnight at 4°C. The next day the beads were washed three times for 10 minutes with immunoprecipitation buffer at 4°C. Then the beads were boiled in 20 µl of SDS-PAGE sample buffer for subsequent analysis by SDS-PAGE and immunoblotting.

Furin inhibition. For furin convertase inhibition studies decanoyl-RVKR-chloromethyl ketone (cmk) (Bachem, Switzerland) was added to S2 cells expressing the full length Tractin construct for a final concentration of 50 mM. The cell were induced with 0.5 mM $CuSO_4$ and grown for 12 h before harvesting and analysis as described above.

Cell adhesion assays

Cell aggregation assays were carried out essentially as described in Hortsch et al. (27). Briefly, S2 cells expressing Tractin constructs were plated in 6-well culture dishes at $1.0x10^6$ cells/ml, induced with 0.5 mM $CuSO_4$, and grown for 12 h before the cells were aggregated for 2 h at room temperature on a shaking platform at 100 rpm. Digital images of the aggregated cells were obtained on a Zeiss Axiovert inverted microscope using a Paultek digital camera. To investigate the role of the interaction between NTF-Fc and CTF in cell adhesion, CTF and ΔIgFN construct expressing S2 cells were plated at $1.0x10^6$ cells/ml. For experimental cultures 100 µl/ml of NTF-Fc lysate obtained from NTF-Fc expressing COS cells as described above were added. For control cell cultures 100 µl/ml of untransfected COS cell lysate were added. The cells were induced with 0.5 mM $CuSO_4$, grown for 12 h and aggregation assays were carried out as described above. For quantification a digital image of a field in the middle of each well was obtained and the number of aggregates containing more than 10 cells were counted. The difference in the number of aggregates found in experimental and control wells was compared using a student's t-test. In some control experiments 100 l/ml of lysate from Fc expressing COS cells was added as described above. That equivalent levels of Fc and NTF-Fc expression was obtained was verified by immunoblotting and labeling with Fc-antibody.

For homophilic interaction assays Tractin transfected S2 cells were labeled with DiI

(Molecular probes, Eugene, OR). The cells were incubated with 2 µl of 2 mg/ml DiI solution for each ml of culture medium at room temperature for 1 h before the cells were washed 3 times with fresh medium to remove excess dye. Untransfected S2 cells was labeled with DiO using Vybrant DiO cell-labeling solution (Molecular probes). 5 µl of the DiO solution were added for each ml of culture medium at room temperature for 1 h before the cells were washed three times with fresh medium. DiI and DiO labeled cells were mixed at 1:1 to a final concentration of $1.0x10^6$ cells/ml. The cell mixture were induced with 0.5 mM $CuSO_4$, grown for 12 h and aggregation assays carried out as described above. Images of the labeled preparations were obtained on a Zeiss Axioskop equipped with the appropriate filter sets and a Paultek digital camera.

Immunohistochemistry

Tractin expressing S2 cells were affixed to polylysine coated slides and fixed for 2 h in 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The slides were incubated 3 h at room temperature with diluted antibody in PBS containing 0.4% Triton X-100 and 0.005% sodium azide, washed in PBS with 0.4% Triton X-100, and incubated with FITC-conjugated goat anti-mouse antibody (ICN, 1:200 dilution). After 3 washes in PBS the fluorescently labeled preparations were mounted in glycerol with 5% *n*-propyl gallate. A confocal series of images for each of the labeled preparations were obtained with a Leica confocal TCS NT microscope at 1 µm intervals using the appropriate laser lines and filter sets.

RESULTS

Figure 1A shows a diagram of the domain structure of Tractin. It contains 6 Ig-domains, 4 FNIII-like domains, an acidic domain, 12 repeats of a novel collagen-like proline- and glycine-rich sequence motif, a transmembrane domain, and an intracellular tail with an

ankyrin and a PDZ-domain binding motif (21, 22). The predicted molecular weight of Tractin is 198 kDa; however, antibodies to the NH_2-terminal sequence of Tractin recognize only a 130 kDa glycosylated fragment on standard immunoblots (Fig. 1B). Furthermore, antibodies to the fourth FNIII- and acidic domain and the PGYG-repeat domain (mAb 3A11, mAb 1H4) both recognize a doublet of bands of approximately 165 and 185 kDa whereas an antibody to the intracellular domain (mAb 3A12) recognizes only the higher 185 kDa band (Fig. 1B). This and observations with other domain-specific antibodies suggest that Tractin is cleaved at two sites (Fig. 1A, arrows), one in the third FNIII-domain, and one just proximal to the transmembrane domain (22). Previously, a full length version of the Tractin protein has not been observed on immunoblots of leech central nervous system proteins (21, 22); however, on greatly overloaded gels the same faint band corresponding to the expected size for the unprocessed protein can be detected with different domain specific antibodies (Fig. 1C). The several hundred fold ratio of cleaved fragments to full length protein indicate that Tractin is constitutively posttranslationally processed in the leech central nervous system.

Expression and processing of Tractin in the S2 cell line

To study the processing of Tractin and to determine the ability of Tractin to mediate homophilic and/or heterophilic interactions we transfected *Drosophila* S2 cells with Tractin expression constructs under the control of a metallotheionin promoter. Figure 2 shows diagrams of the full length and the various truncated Tractin constructs with COOH-terminal V5 epitopes employed in these studies. In addition, we generated an Fc-fusion construct of the NH_2-terminal fragment (NTF) from the starting methionine to the first cleavage site (Fig. 2). Stably transfected S2 cell lines were obtained for the full length construct as well as for the deletion constructs. Interestingly, the cell line expressing full length Tractin cleaves Tractin in a pattern identical to that observed in leech nerve cords (compare Fig. 3 and Fig.

1B). In immunoblots of cell lysate containing cell membranes, antibodies to the COOH-terminal part of Tractin (3A11 and V5) recognize a 180 kDa band whereas antibodies to the NH_2-terminal fragment (Laz6-56) recognize a 110 kDa band (Fig. 3A). As a consequence of the artificial overexpression in the cell culture the full length protein migrating at approximately 290 kDa is relatively more abundant under these conditions than in the *in vivo* situation (Fig. 3A). The 110 kDa NH_2-terminal fragment is found both in the medium and cell lysate (Fig. 3A and B). In contrast, the 160 kDa band is only found secreted into the medium and is not recognized by antibodies to either the NH_2-terminal fragment or the intracellular domain (Fig. 3B). That the molecular masses of these bands do not precisely correspond to those from leech nerve cords is due to differences in glycosylation in the S2 cell line and the added V5 tag.

The migration on SDS-PAGE of the doublet of bands from leech central nervous system proteins recognized by Tractin COOH-terminal domain specific antibodies of 165 and 185 kDa (Fig. 1B) are much higher than the 76 and 98 kDa predicted for these bands based on their amino acid sequence. This discrepancy can either be due to homo- and/or heterodimer formation through the formation of SDS resistant covalent bonds or to anomalous gel migration (22). In order to distinguish between these possibilities we compared the migration on SDS-PAGE of Tractin constructs expressed in the S2 cell line where different COOH-terminal sequences had been deleted (Fig. 4). From this analysis we found that when the acidic and the PGYG domains were deleted gel migration of the peptides were considerably closer to the molecular mass predicted from their amino acid sequence and that the acidic and PGYG domain when present together accounted for 50-65 kDa of the anomalous gel migration (Fig. 4A and B). This observation was confirmed by a GST fusion protein construct expressed in bacteria containing only the acidic and PGYG domains which also migrated with the molecular mass of a peptide 63 kDa larger than the predicted 79 kDa

(Fig. 4C). Taken together these data suggests that homo and/or heterodimer formation through SDS-resistant covalent bonds as previously proposed (22) are unlikely.

Tractin is constitutively cleaved by a furin-like convertase in the third FNIII-domain

Our data from immunoblots with domain specific antibodies (Fig. 1) suggest that Tractin is constitutively cleaved in the ectodomain. Constitutive processing of precursor proteins are often mediated by furin convertases which are calcium-dependent proteases mainly localized in the trans-Golgi network and are ubiquitously expressed by eukaryotic cells (14). In addition, Tractin contains a dibasic sequence conforming to the consensus cleavage site for furin convertases, RxR/KR (14), in the third FNIII-domain. To investigate the possibility of furin convertase dependent cleavage of Tractin we applied the furin inhibitor decanoyl-RVKR-chloromethyl ketone (cmk) (28-30) to full-length Tractin expressing S2 cells for 12 h at a final concentration of 50 mM. As shown in Figure 5A and B incubation with cmk furin inhibitor prevented the generation of the 180 and 110 kDa polypeptides. This observation indicates that these polypeptides, directly or indirectly, are the result of furin mediated proteolytic processing. To test whether the consensus furin site indeed is a site of cleavage we introduced a RKRRSR to AAAASA mutation into the full-length Tractin construct and expressed it in S2 cells. Immunoblot analysis of cell lysate from these cells show that processing at this site of the mutated Tractin construct was completely abolished. Thus, these data indicate that tractin is likely to be cleaved at the RKRRSR sequence in the third FNIII-domain. To test the implication of furin itself in this process we tested the processing of the full length Tractin construct in LoVo cells, a cell line established from a human colon carcinoma that expresses no functional furin (15, 31). Expression of Tractin in the mammalian HEK293T cell line served as a control. Figure 6 shows the results of transiently expressing Tractin in these cell lines. Both the 1H4 and Laz6-56 antibodies

detect the expected cleavage products on immunoblots of cell lysate from the furin containing HEK293T cells whereas no cleaved polypeptides are detectable in the LoVo cells that lack furin. These results support the hypothesis that Tractin processing at the first cleavage site is furin dependent. To further examine whether full-length Tractin is expressed at the cell surface we treated Tractin expressing S2 cells with biotin succinimidyl-ester or mock-treated them without adding biotin. The cells were lysed and the protein extracts immunoprecipitated by Tractin 1H4 antibody. Subsequently, the 1H4 immunoprecipitates were separated by SDS-PAGE and labeled with either 1H4 or anti-biotin antibody on immunoblots. While both full-length Tractin and the COOH-terminal fragment could be detected with 1H4 antibody only the transmembrane fragment was labeled with anti-biotin antibody (Fig. 7). This result suggests that the COOH-terminal cleavage product but not the full-length precursor are present at the cell surface.

Membrane tethering of the cleaved NH_2-terminal Tractin fragment

The above studies provide evidence that the NH_2-terminal fragment of Tractin is constitutively cleaved in the trans-Golgi network yet it is known from immunocytochemistry and immunoelectron microscopy that it is localized to the surface of axons (32, 33). This raises the question of how it is tethered to the membrane. One possibility is that it is interacting with integrins through its RGD integrin-binding motif just upstream of the first cleavage site (21). Another is that it binds to the transmembrane fragment of Tractin after cleavage. We explored the latter possibility by performing cross-immunoprecipitation experiments with Tractin domain specific antibodies of homogenates from S2 cells stably transfected with a full-length V5-tagged Tractin construct. Figure 8A shows that on immunoblots of immunoprecipitations with the Tractin NH_2-terminal mAb Laz6-56 the transmembrane Tractin fragment is detected as a 180 kDa band by the mAbs, 3A11, 1H4,

and V5. Conversely, on immunoblots of homogenate from these cells immunoprecipitated by V5 antibody to the COOH-terminal fragment the NH_2-terminal fragment is detected by Laz6-56 antibody as a 110 kDa band (Fig. 8B). The 1H4 mAb co-immunoprecipitates the 110 kDa NH_2-terminal fragment as detected by mAb Laz6-56 (Fig. 8C, lane 1) from the medium of Tractin transfected S2 cells in addition to the secreted 160 kDa fragment (Fig. 8C, lane 2). The 110 kDa band is not immunoprecipitated from non-Tractin expressing control S2 cells (Fig. 8C, lane 3). Consistent with these findings the 110 kDa NH_2-terminal containing fragment but not the 160 kDa middle fragment of Tractin is found in the cell lysate which contains cell membranes (Fig. 3A). Thus, the cleavage fragments of Tractin interact with each other and this interaction may provide a means for the NH_2-terminal fragment to be tethered to the membrane. To test whether this interaction of the Tractin fragments also occurs in the leech nervous system we performed cross-immunoprecipitation experiments with the Laz6-56 and 1H4 mAbs on protein extracts from leech nerve cords. As indicated by the immunoblots in Fig. 9A and B both the Laz6-56 and 1H4 antibodies can co-immunoprecipitate the transmembrane as well as the cleaved NH_2-terminal secreted fragment demonstrating that the two fragments are likely to interact *in vivo*.

To identify the domain of the transmembrane fragment of Tractin responsible for binding the NH_2-terminal fragment we tested the ability of various truncated transmembrane expression constructs to interact with the NH_2-terminal fragment (Fig. 10). Tractin NH_2-terminal fragment obtained from the medium of stably full-length Tractin expressing S2 cells was bound to Laz6-56 coated protein G beads and incubated with homogenate of cell lysate from ΔIg/FN, ΔIg/FN1-3, or CTF transiently transfected S2 cells. Subsequently, the protein G beads were spun down, washed, and assayed on immunoblots for the presence of the V5-tagged transmembrane deletion constructs with V5 antibody. In all three constructs the entire sequence NH_2-terminal to the furin cleavage site located in the middle of the third FNIII-

domain was deleted. In addition, the remaining part of the third FNIII-domain was deleted in the ΔIg/FN1-3 construct and the fourth FNIII-domain was further deleted in the ΔIg/FN construct. Figure 10 shows that of the three deletion constructs only the CTF construct was detected on the immunoblots with V5 antibody. These data suggest that the interaction between the NH_2-terminal cleavage fragment and the transmembrane fragment is mediated by sequences located in the third FNIII-domain just distal to the furin cleavage site.

Homophilic adhesion of Tractin expressing S2 cells

To determine the ability of Tractin to mediate homophilic and/or heterophilic interactions we transiently and stably transfected *Drosophila* S2 cells with Tractin expression constructs. Among the stably transfected S2 cell lines one expressed a full length construct with a COOH-terminal V5 epitope and one expressed a truncated Tractin construct (ΔAc/PGYG) encompassing sequence from the first cleavage-site in the third FNIII domain to the COOH-terminal end but lacking the acidic and the collagen-like domains (Fig. 2). S2 cells are ideal for adhesion interaction assays since untransfected cells are non-adhesive (Fig. 11A) and do not express any known adhesion molecules (27). When S2 cells are induced with the full length Tractin construct it leads to cell aggregation (Fig. 11B). This aggregation is not observed in cells transfected with the CTF construct (Fig. 12A) but is still present when the acidic and PGYG domains are deleted (Fig. 11C). These data suggest that the cell adhesive properties reside within sequences of the NH_2-terminal domain of Tractin. Expression of Tractin in the transfected cell lines was confirmed by confocal imaging of Tractin antibody labeled cells (Fig. 11D) and by immunoblot analysis (data not shown). To directly test whether the induced interaction was homophilic we labeled untransfected S2 cells with DiO (green) and Tractin transfected S2 cells with DiI (red). Equal numbers of DiO and DiI labeled cells were mixed and grown for 12 h. As shown in Fig. 11E the Tractin

transfected S2 cells formed pure aggregates of DiI labeled cells whereas DiO labeled untransfected S2 cells were scattered and not part of the aggregates. These data suggest that Tractin induces cell adhesion through homophilic interactions mediated by the NH_2-terminal fragments. To further test whether the NH_2-terminal fragment is necessary for cell adhesion we added NTF-Fc constructs to S2 cells transfected with Tractin CTF. S2 cells induced to express Tractin CTF are non-adhesive (Fig. 12A); however, addition of NTF-Fc to the medium results in aggregation (Fig. 12B). In control experiments where Fc-fragment only was applied to the medium of the CTF expressing S2 cells no such aggregation were observed (data not shown). We quantified NTF-Fc induced interaction by counting the number of aggregates containing more than ten cells in experimental versus control wells. In thirteen such experiments control wells had 1.7±1.3 aggregates whereas experimental wells in which NTF-Fc had been added had 21.6±8.2 aggregates. This difference is statistically significant on the $p<0.001$ level (student's t-test). The induced aggregation is a consequence of specific interactions of Tractin NTF-Fc with FNIII domains 3.5-4 since S2 cells transfected with a COOH-terminal construct where these sequences were deleted do not become adhesive and do not form aggregates in the presence of the NTF-Fc construct (Fig. 12D). These findings indicate that tethering of exogeneously provided Tractin NH_2-terminal fragment to S2 cells expressing only the COOH-terminal fragment can functionally restore the adhesive properties of Tractin.

DISCUSSION

In this study we provide evidence that the L1-family CAM Tractin is constitutively cleaved in a furin-dependent process and that the full-length precursor protein does not reach the cell surface. This gives rise to an extracellular amino-terminal fragment and a carboxy-

terminal transmembrane fragment that form a heterodimer through non-covalent interactions. This processing is similar to that of the Notch receptor where furin-dependent cleavage in the trans-Golgi network also determines the functional structure of the molecule (15, 17). The cleavage site of Tractin which conforms to the consensus sequence for processing by furin convertases is found at the same location in the third FNIII domain in a majority of L1-family CAMs (3) including mammalian L1, NrCAM, chick NgCAM, and nematode LAD-1 suggesting this cleavage has a conserved function. This site in mammalian L1 has previously been shown to be sensitive to trypsin (34, 35) as well as to plasmin (36, 37) in cell culture studies; however, the enzyme responsible for *in vivo* L1 cleavage has yet to be determined. Our studies raise the possibility that cleavage by furin convertases may be a general mechanism for L1-family CAM processing. While all Tractin molecules are cleaved at the furin site a fraction of the COOH-terminal fragments are additionally cleaved just proximal to the membrane generating a secreted middle fragment. We do not know the identity of the enzyme responsible for this processing in leech; however, evidence has been presented that this cleavage process which also takes place with mammalian L1 is mediated by a disintegrin metalloproteinase (19).

Interestingly, the studies performed with limited trypsination of mammalian L1 in cell culture studies indicated that the NH_2-terminal fragment generated by this treatment is not released after cleavage from the cell surface (35). Based on this observation it was speculated that it remains in a non-covalent association with its complementary transmembrane cleavage partner (35). In the present study we provide direct experimental evidence for this hypothesis. We show that the NH_2-terminal fragment of Tractin expressed in S2 cells can be tethered to the membrane by interaction with sequences in the third FNIII-domain of the transmembrane fragment and that this interaction is necessary for establishing homophilic cell adhesion. The COOH-terminal transmembrane fragment alone did not

promote cell adhesion when expressed in the S2 cells suggesting that trans interactions between these fragments do not occur under physiological conditions. Analysis with truncated constructs demonstrated that sequences just distal to the furin cleavage site of the third FNIII-domain were necessary for binding of the NH_2-terminal domain; however, the sequence or sequences responsible for binding within the NH_2-terminal domain remains to be determined. Previous studies have shown that multiple regions of the NH_2-terminal domain of mammalian L1 can interact with the third FNIII-domain including several of the Ig-domains (35). These findings suggest that several regions of the NH_2-terminal fragment may be involved in the binding to the third FNIII-domain. Recent studies have suggested that the third FNIII-domain in mammalian L1 also plays an important role in homomultimerization leading to trimeric L1 and a concomitant recruitment of integrins by means of cis interactions (37). The L1 trimerization is regulated by ligand interactions of the extracellular domain. Interestingly, the multimerization is abolished by proteolytic cleavage within the third FNIII-domain (37). However, since Tractin is constitutively cleaved at this site multimerization of Tractin by this mechanism is not likely to occur.

An important issue is whether interactions observed in heterologous cell culture systems also take place *in vivo*. We found that both the Laz6-56 and 1H4 antibodies can co-immunoprecipitate the transmembrane as well as the cleaved NH_2-terminal secreted fragment from leech nerve cord extracts strongly indicating that the two fragments also are likely to interact in the nervous system. Interestingly, the secreted middle fragment generated from proteolysis at the furin site as well as just distal to the transmembrane segment is also located to the surface of neurons and axons (22). A likely explanation for this membrane localization is that the middle fragment may interact heterophilically with other molecules. It is well established that many L1-family CAMs can interact with various extracellular matrix components (1, 38) raising the possibility that the secreted middle

fragment of Tractin may function as a substrate adhesion molecule by incorporation into the extracellular matrix. Such an interaction may be facilitated by the collagen-like properties of the PGYG-domain (22).

Interaction with its own transmembrane cleavage fragment may not be the only mechanism for tethering the NH_2-terminal fragment of Tractin to the membrane as this fragment contains an RGD integrin-binding motif at the beginning of the third FNIII domain (21). Consequently, the possibility exists that Tractin NH_2-terminal fragments are linked to integrins as well as to the Tractin COOH-terminal fragment and in this way may provide a capability of signaling through two different signal transduction systems. This notion is supported by the findings that the shedded ectodomain of mammalian L1 interacts with integrins through a RGD motif located in the sixth Ig-domain and that this interaction may play a distinct functional role in promoting cell migration (19). Thus, controlled posttranslational proteolysis of CAMs may be a general mechanism for enhancing functional diversity by generating cleaved ectodomains that can bind to different molecules involved in distinct signal transduction pathways or extracellular matrix interactions.

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FOOTNOTES

[1]The abbreviations used are: CAM, cell adhesion molecule; mAb, monoclonal antibody; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; GST, glutathione-S-transferase; PMSF, phenylmethylsulfonyl fluoride; FBS, fetal bovine serum; DAB, diaminobenzidine; PAGE, polyacrylamide gel electrophoresis; HRP, horse radish peroxidase; FITC, fluorescein-5-isothiocyanate; cmk, decanoyl-RVKR-chloromethyl ketone.

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FIGURE LEGENDS

Fig. 1. **Domain specific Tractin antibodies.** (A) Diagram of the Tractin protein. The protein sequence is organized into six Ig-domains, four FNIII-domains, an acidic domain, a PGYG-repeat containing domain which is collagen-like, a transmembrane domain (TM), and a cytoplasmic domain with an ankyrin-binding and a PDZ-binding (SxV) motif. The two putative proteolytic cleavage sites in the third FNIII-domain (cleavage site I) and between the PGYG-repeat and transmembrane domains are indicated by arrows. The monoclonal antibodies 4G5, 3A11, 1H4, and 3A12 were made to fusion proteins or peptides of Tractin sequences as indicated by the black horizontal bars. mAb Laz6-56 (6-56) recognizes the NH_2-terminal fragment of Tractin. The predicted molecular mass of the fragments generated by proteolytic cleavage at site I and II is shown on the line below the diagram. (B) Immunoblots of *Haemopis* nerve cord proteins labeled with Tractin domain specific antibodies. Antibody to the NH_2-terminal fragment (4G5) recognized a 130 kDa band, antibody to the fourth FNIII and/or acidic domain (3A11) recognized a doublet of bands, whereas antibody to the cytoplasmic domain (3A12) only recognized the high band of the doublet. (C) Immunoblots of *Haemopis* nerve cord proteins after separation by SDS-PAGE and labeled with mAb 1H4 and Laz6-56. On these immunoblots of intentionally overloaded lanes full-length Tractin are recognized by both antibodies as a weak band (arrow) migrating with a relative molecular mass of 290 kDa. In (B) and (C) the migration of molecular mass markers in kDa is indicated in grey.

Fig. 2. **Diagrams of Tractin deletion and expression constructs.** The full-length and the COOH-terminal deletion constructs have an in frame V5-tag (V5) whereas the NTF-construct was fused to an Fc-tag (Fc). The different domains of Tractin is indicated above

the figure: Ig (immunoglobulin domains); FN (FNIII-domains); Ac (acidic domain); PGYG (PGYG collagen-like domain); TM (transmembrane domain).

Fig. 3. **Proteolytic processing of a full length Tractin construct in stably transfected S2 cells.** (A) Immunoblot of S2 cell lysate that includes cell membranes. The Tractin construct is tagged with the V5 epitope at the COOH-terminal end. Full-length Tractin is detected as an approximately 280 kDa band with both Laz6-56, 3A11, and V5 antibody, the COOH-terminal transmembrane fragment as a 180 kDa band by 3A11 and V5 antibody, whereas the NH_2-terminal fragment is detected as a 110 kDa band by the mAb Laz6-56. That the NH_2-terminal fragment is found in the cell lysate suggests that it is tethered to the cell surface. (B) Immunoblot of cell medium from S2 cells expressing full-length Tractin. In the cell medium the secreted middle fragment is detected as a 160 kDa band by mAb 3A11 whereas the secreted form of the NH_2-terminal fragment is detected as a 110 kDa band by mAb Laz6-56. No V5-tagged fragments of Tractin is detected in the cell medium by V5 antibody. The migration of molecular mass markers in kDa is indicated in grey.

Fig. 4. **Relative migration of Tractin deletion constructs and domains separated by SDS-PAGE.** Immunoblots of cell lysate from S2 cells expressing full length Tractin, ΔPGYG, ΔAc, and ΔAc/PGYG constructs, respectively, were labeled with V5 antibody in (A) and with Laz6-56 antibody in (B). The predicted molecular mass of the full length Tractin construct is 200 kDa; however, it migrates as an approximately 290 kDa protein relative to molecular mass markers on SDS-PAGE (lane 1 and 5). The majority of this anomalous gel migration is due to sequences in the COOH-terminal Tractin fragment as the NH_2-terminal fragment migrates close to its predicted size of 100 kDa (arrow in (B)).

Deleting the Ac and PGYG domains individually does not significantly change the anomalous gel migration (lane 2, 3, 6, and 7); however, when both the acidic and PGYG domains are deleted the relative migration of the resulting peptides were 50-65 kDa closer to the migration predicted from their amino acid sequence (lane 4 and 8). (C) The relative migration of a GST-fusion protein containing only the Ac and PGYG domains detected on an immunoblot with mAb 1H4. The fusion protein migrates as a 142 kDa protein. This migration is 63 kDa larger than that predicted for a peptide with a molecular mass of 79 kDa. The migration of molecular mass markers in kDa is indicated in grey.

Fig. 5. **Furin dependent cleavage of Tractin expressed in S2 cells.** (A) and (B) Expression of full-length Tractin were induced with $CuSO_4$ in stably transfected S2 cells for 12 h in the absence (cmk-) or presence (cmk+) of the furin convertase inhibitor decanoyl-RVKR-chloromethyl ketone (cmk). The cell lysate from the cells were separated by SDS-PAGE, immunoblotted, and Tractin detected with V5 antibody in (A) and with mAb Laz6-56 in (B). In the presence of furin inhibitor (cmk+) neither antibody was able to detect any of the Tractin cleavage fragments obtained without furin inhibitor (cmk-). (C) Site directed mutagenesis of the consensus furin convertase cleavage site RKRRSR in Tractin. The lysines and the arginines at the cleavage site were mutated into alanines by PCR. When this construct was expressed in S2 cells no cleavage fragments, only full length Tractin, was detected by the antibodies Laz6-56, 3A11, and V5 on immunoblots of cell lysate. The migration of molecular mass markers in kDa is indicated in grey.

Fig. 6. **Tractin processing in LoVo cells. LoVo cells do not express any functional furin convertase.** In LoVo cells transiently transfected with a full length Tractin construct no Tractin cleavage fragment can be detected on immunoblots of cell lysate by

either mAb 1H4 or Laz6-56 (lane 2 and 4). In contrast, in control furin expressing HEK293T cells (293T) transiently transfected with the same construct the expected Tractin cleavage fragment is labeled by the respective antibodies (lane 1 and 3). The migration of molecular mass markers in kDa is indicated in grey.

Fig. 7. **Full length Tractin is not present at the cell surface in S2 cells.** Tractin expressing S2 cells were treated with biotin succinimidyl-ester (biotin +) or mock-treated without adding biotin (-). The cell lysate from both groups of cells was immunoprecipitated by 1H4 antibody followed by SDS-PAGE and immunoblotting. The immunoblots were labeled with the Tractin antibody 1H4 (lane 1 and 2) and with antibody to biotin (lane 3 and 4). While both full-length Tractin and the COOH-terminal fragment could be detected with 1H4 antibody only the transmembrane fragment was labeled with anti-biotin antibody in biotin treated S2 cells (lane 3). The migration of molecular mass markers in kDa is indicated in grey.

Fig. 8. **The NH_2-terminal fragment of Tractin interacts with the COOH-terminal fragment in co-immunoprecipitation assays.** (A) Immunoblots of cell lysate from S2 cells stably transfected with a full length Tractin construct and immunoprecipitated with the Tractin NH_2-terminal specific mAb Laz6-56. The transmembrane Tractin COOH-terminal fragment is detected as a 180 kDa band by the 3A11, 1H4, and V5 mAbs. (B) On immunoblots of cell lysate from Tractin expressing S2 cells immunoprecipitated by V5 antibody the NH_2-terminal Tractin fragment is detected by Laz6-56 antibody as a 110 kDa band. (C) The 1H4 mAb co-immunoprecipitates the 110 kDa NH_2-terminal fragment as detected by mAb Laz6-56 (lane 1) from the medium of Tractin expressing S2 cells S2/Tractin) in addition to the secreted middle fragment of 160 kDa (lane 2). The 110 kDa

band is not immunoprecipitated from non-Tractin expressing control S2 cells (lane 3).

Fig. 9. **The NH_2-terminal fragment of Tractin extracted from leech nerve cords interacts with the COOH-terminal Tractin transmembrane fragment.** (A) and (B) Immunoblots of *Haemopis* nerve cord proteins immunoprecipitated with mAbs 1H4 and Laz6-56, respectively, and compared to nerve cord lysate (lysate). The immunoblots were labeled with mAb Laz6-56 in (A) and with mAb 1H4 in (B). Both the Laz6-56 and 1H4 antibodies can co-immunoprecipitate the transmembrane 185 kDa as well as the cleaved 130 kDa NH_2-terminal fragment demonstrating that the two fragments are likely to interact *in vivo*. The migration of molecular mass markers in kDa is indicated in grey.

Fig. 10. **The NH_2-terminal fragment of Tractin interacts specifically with the third FNIII domain of the COOH-terminal transmembrane fragment.** Tractin NH_2-terminal fragment was bound to Laz6-56 coated protein G beads and incubated with homogenate of cell lysate from ΔIg/FN, ΔIg/FN1-3, or CTF Tractin construct expressing S2 cells. Subsequently, the immunobeads were pelleted and assayed on immunoblots for the presence of the V5-tagged transmembrane deletion constructs with V5 antibody. Of the three deletion constructs only the CTF construct was detected on the immunoblots with V5 antibody indicating that the interaction between the NH_2-terminal cleavage fragment and the transmembrane fragment is mediated by sequences located in the third FNIII-domain. The migration of molecular mass markers in kDa is indicated in grey.

Fig. 11. **Aggregation of Tractin expressing S2 cells.** (A) Non-induced control S2 cells do not adhere. (B) Induction of expression of a full length Tractin construct in stably transfected S2 cells leads to cell adhesion and the formation of large cell aggregates (arrows).

(C) Cell aggregates are still present when S2 cells express the ΔAc/PGYG Tractin construct where the acidic and the PGYG domains are deleted. (D) Confocal image of an aggregate of Tractin expressing S2 cells labeled with mAb Laz6-56. The antibody labeling is predominantly associated with the cell surface. (E) Homophilic adhesion (arrow) of Tractin transfected S2 cells labeled with DiI (red) in a mixture of untransfected S2 cells labeled with DiO (green).

Fig. 12. **The NH_2-terminal Tractin fragment is necessary for cell aggregation through interaction with the FNIII-domains of the transmembrane fragment.** (A) S2 cells expressing the CTF-contruct do not adhere. (B) Addition of NTF-Fc fusion protein to the medium of CTF-construct expressing S2 cells induces adhesion and cell aggregation (arrows). (C) S2 cells expressing the ΔIg/FN-contruct do not adhere. (D) Addition of NTF-Fc fusion protein to the medium of ΔIg/FN-contruct expressing S2 cells does not induce cell aggregation.

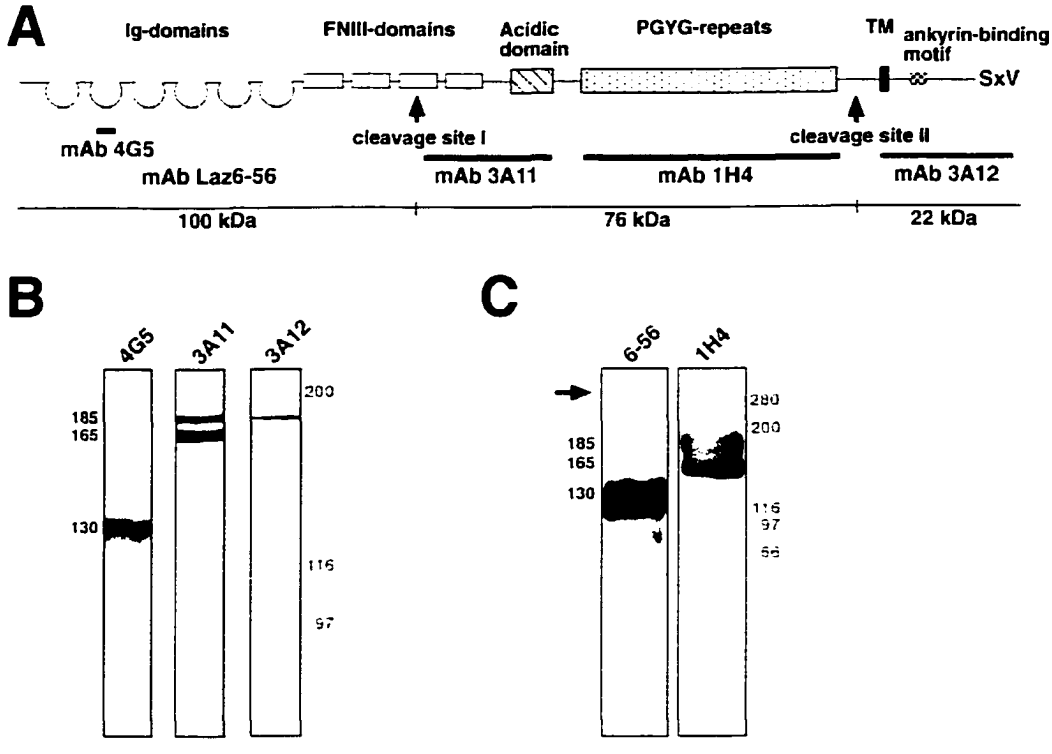

A
Ig-domains
FNIII-domains
Acidic domain
PGYG-repeats
TM
ankyrin-binding motif
SxV
mAb 4G5
mAb Laz6-56
cleavage site I
mAb 3A11
mAb 1H4
cleavage site II
mAb 3A12
100 kDa
76 kDa
22 kDa
B
4G5
3A11
3A12
200
185
165
130
116
97
C
6-56
1H4
280
200
185
165
130
116
97
66

Fig. 1

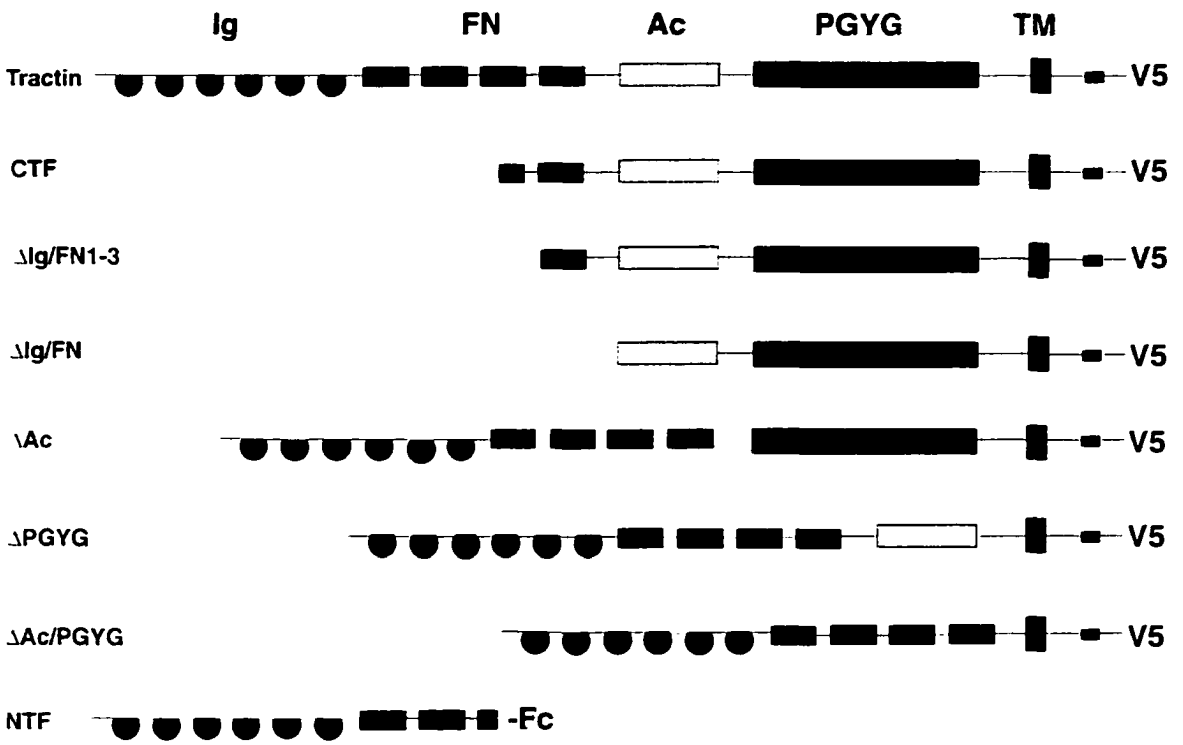

Ig
FN
Ac
PGYG
TM
Tractin
V5
CTF
V5
ΔIg/FN1-3
V5
ΔIg/FN
V5
ΔAc
V5
ΔPGYG
V5
ΔAc/PGYG
V5
NTF
-Fc

Fig. 2

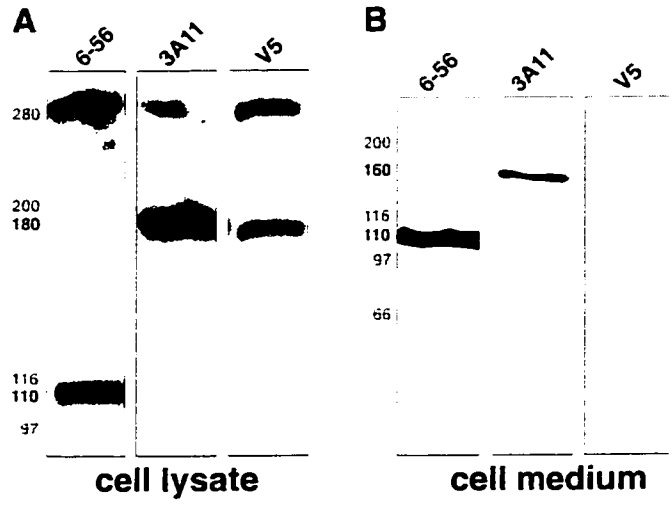

A
6-56
3A11
V5
280
200
180
116
110
97
cell lysate
B
6-56
3A11
V5
200
160
116
110
97
66
cell medium

Fig. 3

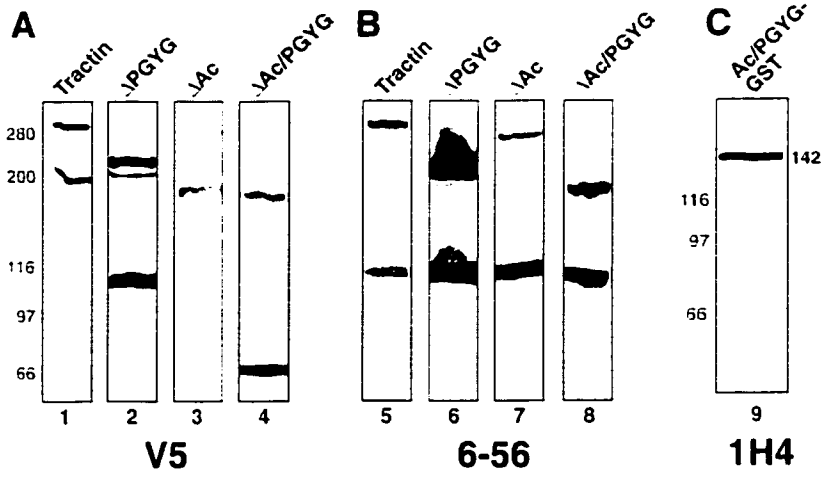

A
Tractin
ΔPGYG
ΔAc
ΔAc/PGYG
280
200
116
97
66
1
2
3
4
V5
B
Tractin
ΔPGYG
ΔAc
ΔAc/PGYG
5
6
7
8
6-56
C
Ac/PGYG-GST
142
116
97
66
9
1H4

Fig. 4

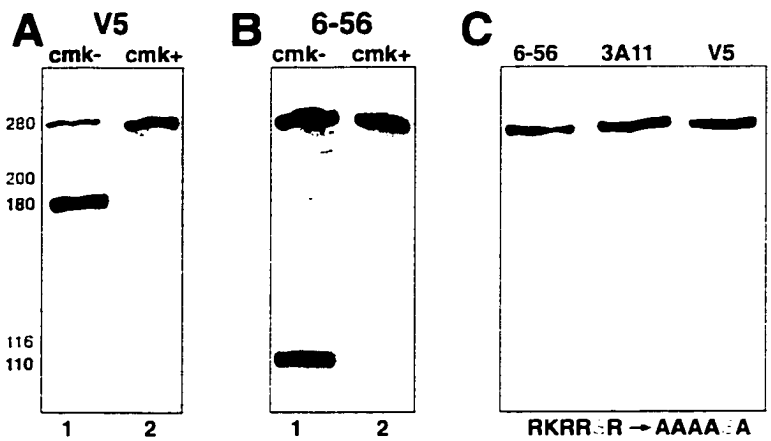
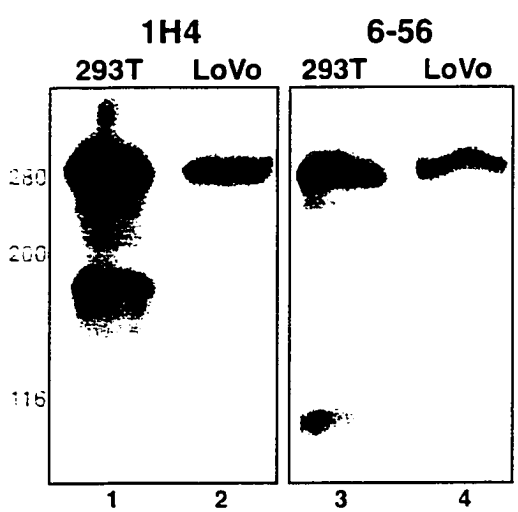

A
V5
cmk-
cmk+
280
200
180
116
110
1
2
B
6-56
cmk-
cmk+
1
2
C
6-56
3A11
V5
RKRR→AAAA

Fig. 5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6-56
293T
LoVo
293T
LoVo
280
200
116
1
2
3
4

Fig. 6

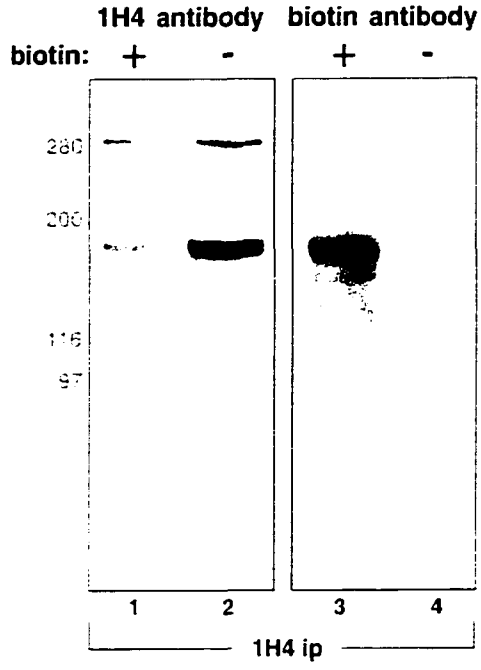
1H4 antibody
biotin antibody
biotin: + - + -
280
200
116
97
1
2
3
4
1H4 ip

Fig. 7

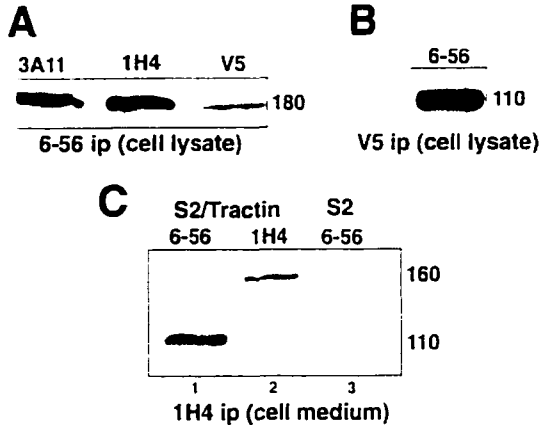
A
3A11
1H4
V5
180
6-56 ip (cell lysate)
B
6-56
110
V5 ip (cell lysate)
C
S2/Tractin
S2
6-56
1H4
6-56
160
110
1
2
3
1H4 ip (cell medium)

Fig. 8

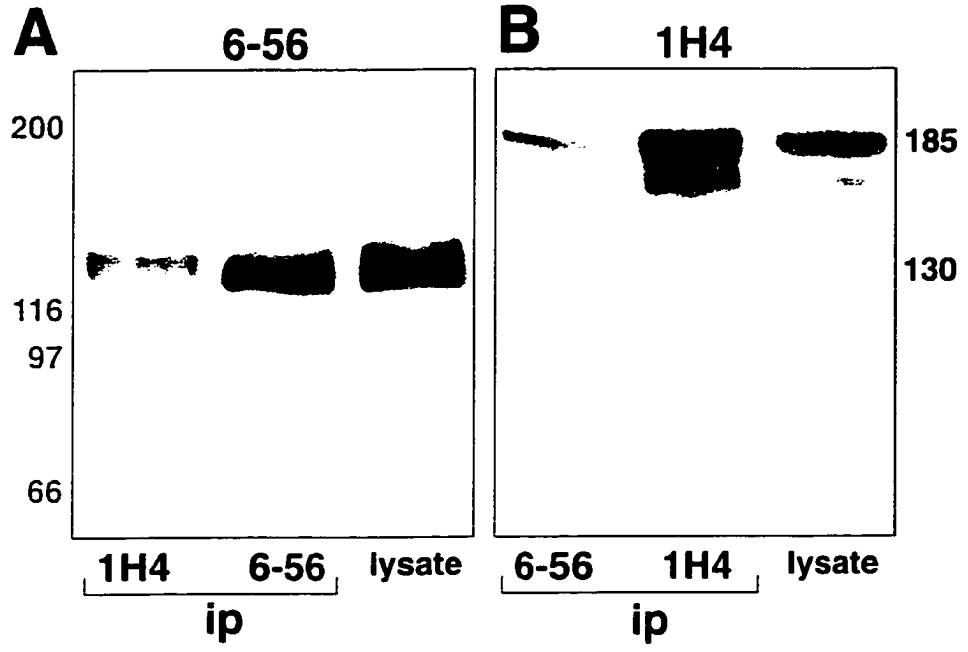
A
6-56
B
1H4
200
116
97
66
185
130
1H4
6-56
lysate
ip
6-56
1H4
lysate
ip

Fig. 9

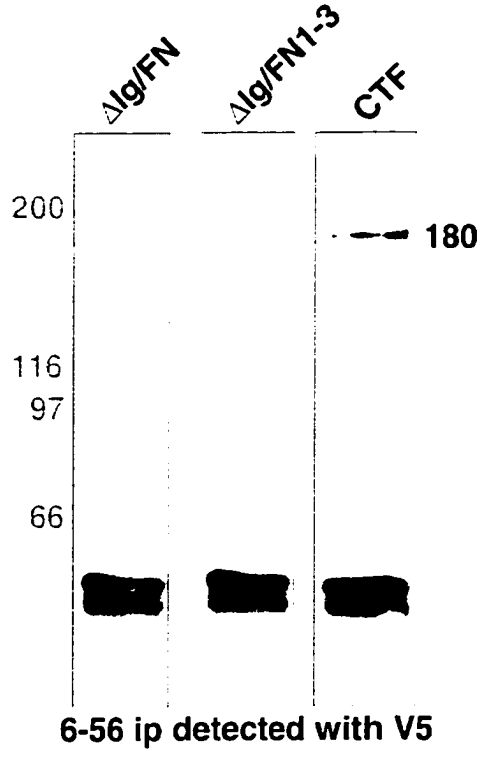
ΔIg/FN
ΔIg/FN1-3
CTF
200
116
97
66
180
6-56 ip detected with V5

Fig. 10

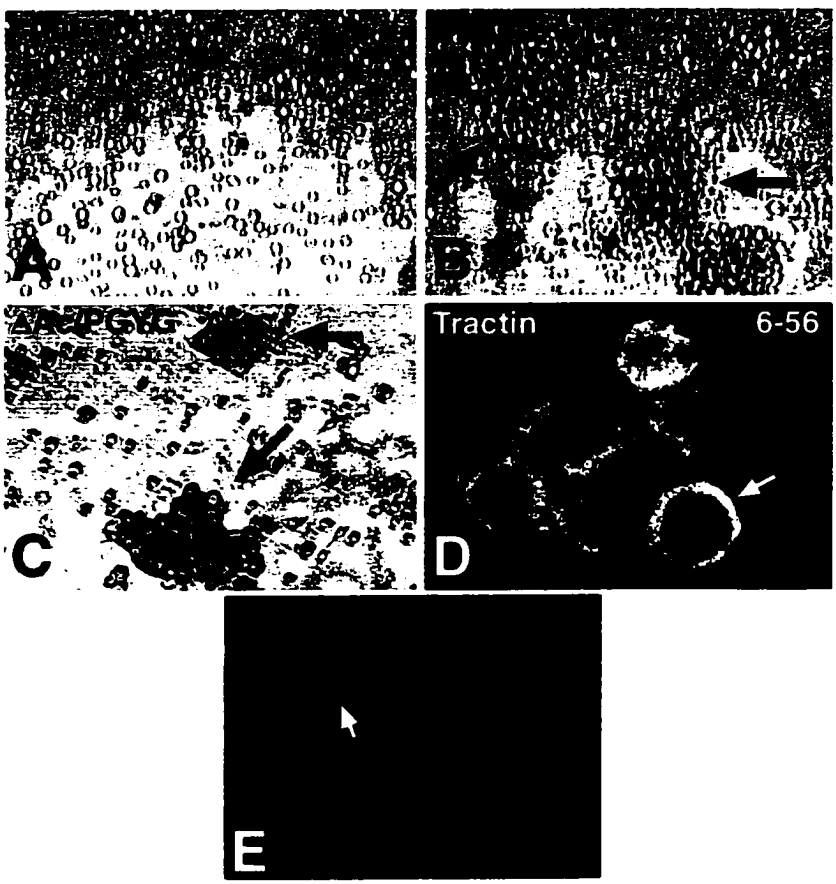

Fig. 11

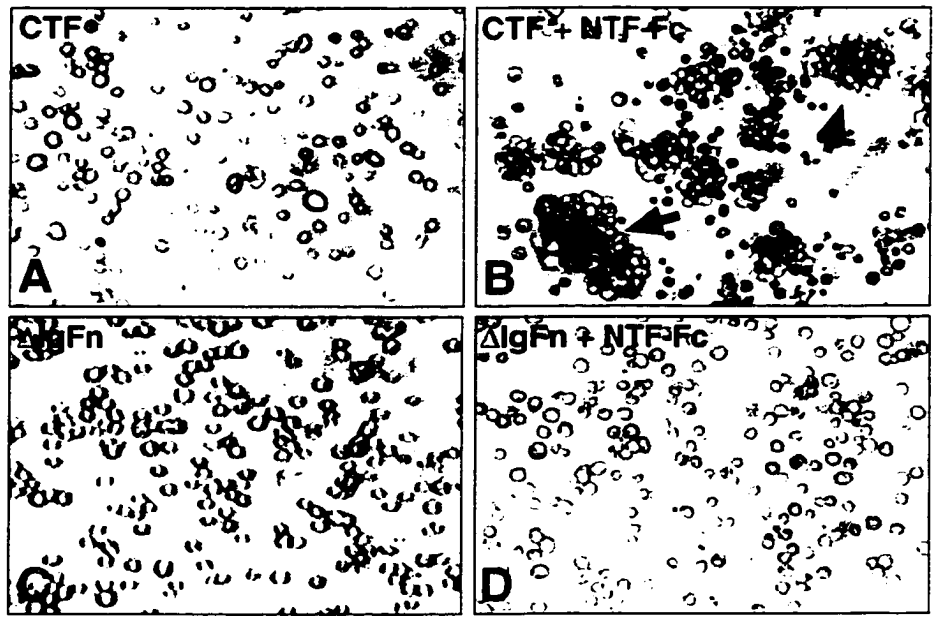

Fig. 12

GLIARIN AND MACROLIN, TWO NOVEL INTERMEDIATE FILAMENT PROTEINS SPECIFICALLY EXPRESSED IN SETS AND SUBSETS OF GLIAL CELLS IN LEECH CENTRAL NERVOUS SYSTEM

A paper published in the Journal of Neurobiology

Yingzhi Xu*, Brian Bolton*, Birgit Zipser+, John Jellies†,
Kristen M. Johansen* and Jørgen Johansen*

ABSTRACT

Using monoclonal antibodies we have identified two novel intermediate filament (IF) proteins, Gliarin and Macrolin, which are specifically expressed in the central nervous system of an invertebrate. The two proteins both contain the coiled-coil rod domain typical of the superfamily of IF proteins flanked by unique N- and C-terminal domains. Gliarin was found in all glial cells including macro- and microglial cells whereas Macrolin was expressed in only a single pair of giant connective glial cells. The identification of Macrolin and Gliarin together with the characterization of the strictly neuronal IF protein Filarin in leech central nervous system demonstrate that multiple neuron and glial specific IFs are not unique to the vertebrate nervous system but are also found in invertebrates. Interestingly, phylogenetic analysis based on maximum parsimony indicated that the presence of neuron and glial cell specific IFs in coelomate protostomes as well as in vertebrates is not of

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monophyletic origin but rather represents convergent evolution and appears to have arisen independently.

INTRODUCTION

Intermediate filaments (IFs) are cytoskeletal proteins that constitute a diverse multigene family that contains more than 50 different IF genes (Albers and Fuchs, 1992). The IFs are differentially expressed within nearly all cell types and serve as mechanical integrators of the cytoplasm that function to resist mechanical stress (Fuchs and Cleveland, 1998). Despite their diversity all IF proteins share common structural features which include an α-helical rod domain defined by regions of heptad repeats in which the 1st and 4th residue usually are hydrophobic or nonpolar (Steinert and Roop, 1988). The rod domain is flanked by variable N- and C-terminal domains which are responsible for a major part of the structural heterogeneity of IF proteins (Steinert and Roop, 1988). Based on similarities in sequence structure and intron placement vertebrate IFs have generally been divided into 6 classes (Steinert and Roop, 1988; Lendahl et al., 1990; Albers and Fuchs, 1992): Type I and II keratins; Type III cytoplasmic IFs; Type IV neurofilaments; Type V nuclear lamins; and Type VI nestins. In addition to the 6 types of vertebrate IFs, an increasing number of invertebrate IFs have also been cloned and characterized (Weber et al., 1988; 1989; Szaro et al., 1991; Tomarev et al., 1993; Dodemont et al., 1994; Johansen and Johansen, 1995; Bovenschulte et al., 1995). Interestingly, invertebrate IFs share a feature with the nuclear lamins of having 6 heptad repeats in the rod domain which are not found in vertebrate cytoplasmic IFs raising questions as to the evolutionary history of IF proteins (Osborn and Weber, 1986; Weber et al., 1988; Steinert and Roop, 1988).

We are particularly interested in IFs' role and distribution within the invertebrate nervous system and whether IFs specific to different components of the central nervous

system such as neurons and glia exist. Previously only two types of such IFs, squid brain IF (Szaro et al., 1991; Way et al., 1992) and Filarin (Johansen and Johansen, 1995), have been demonstrated in the invertebrate central nervous system and they are both selectively expressed by neurons. However, in this study we show that in addition to neurons, IFs can also be found to be specifically expressed in sets and subsets of glial cell types within the leech central nervous system. The leech central nervous system consists of a head and a tail brain and 21 similar segmental ganglia linked to each other by connectives (Muller et al., 1981). Each segmental ganglion contains about 400 neurons which are relatively large, some being up to 100 μm in diameter (Macagno, 1980). In addition to microglial cells there are four types of giant glial cells present within the central nervous system in each segment (Coggeshall and Fawcett, 1964; Lüthi et al., 1994): 1) one pair of macroglia cells located in the connectives wrap all axons traveling in the two lateral connectives and Faivre's nerve; 2) six packet glial cells envelope the neuronal cell bodies in each ganglion; 3) two large glial cells surround axons and neuronal processes in the neuropil; and 4) two glial cells ensheathe axons in the peripheral nerve roots. Using two new monoclonal antibodies (mAbs) first reported here, 9A8 and 1A11, as well as two previously characterized mAbs, G39 (Lüthi et al., 1994) and Lan3-13 (Flaster and Zipser, 1987; Morrisey and McGlade-McCulloh, 1988), we show that all types of glial cells within the leech central nervous system express a novel IF protein which we have named Gliarin whereas the connective macroglia cell, in addition, specifically expresses a different IF protein which we have named Macrolin.

MATERIALS AND METHODS

Experimental preparations.

For the present experiments we used two different leech species, namely the hirudinid leeches *Hirudo medicinalis* and *Haemopis marmorata*. The leeches were either captured in the wild or purchased from commercial sources. Dissections of nervous tissue were performed in leech saline solutions with the following composition (in mM): 110 NaCl, 4 KCl, 2 $CaCl_2$, 10 glucose, 10 Hepes, pH. 7.4.

Antibodies and ascites production.

Monoclonal antibody production essentially followed the procedure of Zipser and McKay (1981). In short, Balb C mice were immunized at 21 day intervals by intraperitoneal injection with a homogenate of the entire nerve cords from four *Hirudo* leeches. Four days after a boost by intravenous injection of SDS-extracted nerve cords spleen cells of the mice were fused with Sp2 myeloma cells and a number of monospecific hybridoma lines established using standard procedures (Harlow and Lane, 1988). Two of these hybridoma lines, 1A11 and 9A8, were specific for leech glial cells and were selected for further analysis in the present paper. In addition, three previously reported mAbs, G39 (Lüthi et al., 1994), Lan3-13 (Flaster and Zipser, 1987; Morrisey and McGlade-McCulloh, 1988), and Lan3-8 (McKay et al., 1984; Johansen and Johansen, 1995) were used in these studies. Ascites fluids from mAb 1A11 and G39 were obtained by injecting 4 mice for each mAb intraperitoneally with antibody producing hybridoma cells. All the mAbs used in this study show cross-reactivity in both *Hirudo* and *Haemopis* leeches.

Immunocytochemistry.

Dissected leech nerve cords were fixed overnight at 4°C in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4, the connective capsules on the ventral side opened with fine forceps, and the ganglia xylene extracted for better antibody penetration (Zipser and McKay, 1981). The nerve cords were incubated overnight at room temperature in either hybridoma supernatant or diluted ascites fluid of the mAbs 9A8, 1A11, G39, Lan3-8 or Lan3-13 containing 0.4% Triton X-100, washed in PBS with 0.4% Triton X-100, and incubated with HRP-conjugated goat anti-mouse antibody (Bio-Rad, 1:200 dilution). After washing in PBS, the HRP-conjugated antibody complex was visualized by reaction in 3,3'-diaminobenzidine (DAB) (0.03%) and H_2O_2 (0.01%) for 10 min. The final preparations were dehydrated in alcohol, cleared in xylene, and embedded as whole-mounts in Depex mountant. The labeled preparations were photographed on a Zeiss Axioskop using Ektachrome 64T film. The color positives were digitized using Adobe Photoshop and a Nikon Coolscan slide scanner. In Photoshop the images were converted to black and white and image processed before being imported into Freehand (Macromedia) for composition and labeling.

Molecular cloning and sequence analysis.

Ascites fluid from the mAbs 1A11 and G39 and hybridoma supernatant from the Lan3-8 and 9A8 antibodies were used to screen a random primed *Hirudo* central nervous system-enriched cDNA lambda-ZAP II expression library (Huang et al., 1997) essentially according to the procedures of Sambrook et al. (1989) at a density of 30,000 plaque-forming units/150 mm plate. Positive clones were plaque purified and in vivo excised to generate pBluescript phagemids according to the method provided by the manufacturer (Stratagene). Several partial cDNAs were identified by each antibody in these screens. To identify additional clones in order to obtain the full sequence of the cDNAs for Gliarin, Macrolin, and

Hirudo Filarin, the same cDNA library was rescreened using ^{32}P-labeled fragments of the originally identified clones. The fragments were radiolabeled using random priming according to the manufacturer's procedure (Prime-a-Gene kit, Promega) and the library screened using standard procedures (Sambrook et al., 1989).

DNA sequencing was performed using an Applied BioSystem DNA Sequencer 377A at the ISU Nucleic Acid Sequencing Facility using commercially available universal and reverse sequencing primers (Stratagene) or specific primers synthesized at the ISU DNA Sequencing and Synthesis Facility based on the determined sequences. The nucleotide and predicted amino acid sequences were analyzed using the GCG (Genetics Computer Group Package, Version 8, Madison, WI) suite of programs (Devereux et al., 1984). The Gliarin, Macrolin, and Filarin sequences were compared with known and predicted proteins in the SwissProt and Genbank databases using the FASTA and TFASTA programs within the GCG package. In addition, a BLAST search was performed using the NCBI BLAST e-mail server (Altschul et al., 1990) comparing the Macrolin, Gliarin, and Filarin sequences with SwissProt, PIR, and GenPept databases.

Biochemical analysis.

SDS-PAGE was performed according to standard procedures (Laemmli, 1970). Electroblot transfer was performed as in Towbin et al. (1979). For these experiments we used the Bio-Rad Mini PROTEAN II system, electroblotting to 0.2 μm nitrocellulose, and using anti-mouse HRP-conjugated secondary antibody (Bio-Rad) (1:3000) for visualization of primary antibody diluted in Blotto for immunoblot analysis. The signal was developed with DAB (0.1 mg/ml) and H_2O_2 (0.03%) and enhanced with 0.008% $NiCl_2$. The Western blots were digitized using Photoshop software and an Arcus II scanner (AGFA).

Phylogenetic analysis.

Alignments used to produce maximum parsimony trees were generated with the Clustalw version 1.7 program and initially encompassed the rod domain of the IFs analyzed. However, in the final analysis any gaps in the resulting alignments were removed by deleting residues corresponding to the gaps. In the analysis restricted to the cytoplasmic coelomate invertebrate IF sequences the initial alignment consisted of the entire IF sequence before gap removal. Trees were constructed by maximum parsimony using the PAUP computer program version 3.1.1 (Swofford, 1993) on a Power Macintosh G3. All trees were generated by heuristic searches and bootstrap values in percent of 1000 replications (Felsenstein, 1985) are indicated on the bootstrap 50% majority rule consensus trees.

RESULTS

To obtain probes specific to different components of the leech nervous system, panels of mAbs were generated from immunizing mice with a protein homogenate obtained from whole *Hirudo* nerve cords. Two of the resulting mAbs, 9A8 and 1A11, showed immunocytochemical labeling restricted to glial cells in whole-mount preparations of leech nerve cords and ganglia (Fig. 1). While mAb 9A8 labeled all known glial cells including microglia and the four types of giant glial cells (Fig. 1C), labeling of mAb 1A11 was restricted to the connective macroglial cell (Fig. 1A,B). There are only two of these cells per segment as each cell is responsible for wrapping the thousands of axons in each of the paired lateral connectives and one or the other also wraps the axons of the unpaired Faivre's nerve. The connective macroglial cells are truly giant as they can be up to a centimeter long (Fig. 1A). Previously, a mAb, G39, was reported (Lüthi et al., 1994) to have a staining pattern

similar to that of mAb 9A8, whereas the Lan3-13 antibody described by Flaster and Zipser (1987) and by Morrisey and McGlade-McCulloh (1988) was specific to the connective macroglial cell as in the case of mAb 1A11. We therefore compared the labeling of these antibodies with mAbs 9A8 and 1A11 on immunoblots (Fig. 2). In this analysis the labeling of mAb 9A8 was indistinguishable from that of mAb G39 while the labeling of mAb 1A11 was indistinguishable from that of Lan3-13. mAbs G39 and 9A8 both recognize a triplet of bands with the top band having an apparent molecular weight of 70 kDa. On the other hand the mAbs 1A11 and Lan3-13 recognize a doublet with the largest band having an apparent molecular weight of 78 kDa. The lower bands in both cases may represent proteolytic fragments. These data strongly suggest that mAbs 9A8 and G39 recognize the same 70 kDa antigen whereas mAbs 1A11 and Lan3-13 both recognize a different larger 78 kDa antigen. The molecular sizes and the immunocytochemical staining for both antigens made them candidates for being IF proteins. Direct evidence for this has been provided in the case of the mAb G39 antigen from which a partial amino acid sequence, QNQQLSDYEGEISLL, was obtained by Lüthi et al. (1994). This sequence showed 80% homology to a stretch within the rod domain of the squid IF protein NF60 (Szaro et al., 1991). For these reasons and to obtain the complete amino acid sequence of these antigens we screened a *Hirudo* central nervous system expression vector library with the glial cell specific antibodies.

Gliarin. The leech cDNA library was screened independently with both the 9A8 and G39 mAbs resulting in the identification of several hundred partial cDNA clones by each antibody of which 4 and 6, respectively, were selected for further analysis. Subsequently, the cDNA library was rescreened with radiolabeled nucleotide probes generated from the 5' ends of these original cDNA clones. In this way additional independent and overlapping cDNA clones were isolated that encompassed the entire coding sequence. The predicted sequence for the protein identified in both the mAb 9A8 and G39 screen was identical and contained

639 amino acids with a calculated molecular mass of 71,340 Da. In addition, the protein contained the exact QNQQLSDYEGEISLL sequence previously determined from the immuno-affinity purified mAb G39 antigen (Lüthi et al., 1994) confirming the identity of the isolated cDNA. Since the 9A8/G39 antigen is expressed by all glia cells in the leech nervous system we have named it Gliarin. The complete sequence for Gliarin is shown in Fig. 3, and the inferred protein product contains all the defining features of an IF protein (Steinert and Roop, 1988). It has a 96 amino acid N-terminal head domain followed by a α-helical rod domain of 355 residues (Fig. 3) which shows the typical segmentation into subdomains (coils 1A, 1B, 2A, and 2B) separated by short non-helical spacers (linkers L1, L12, and L2). The coiled-coil regions conform with the repeated heptad unit structure $(a,b,c,d,e,f,g)_n$ where a hydrophobic or non-polar amino acid is usually in the a and d position and the other positions are occupied by either polar or charged amino acids (Steinert and Parry, 1985). The C-terminal tail domain consists of 188 amino acids. Coil 1B contains the 6 additional heptad repeats characteristic of nuclear lamins and invertebrate cytoplasmic IFs (Fuchs and Weber, 1994).

Macrolin. Of approximately 4.5 x 10^5 clones screened with the mAb 1A11 two partial antibody-positive cDNA clones were identified. From rescreening with radiolabeled nucleotide probes from the 5' and 3' ends of the original cDNA clones, additional independent and overlapping cDNA clones were isolated that covered the entire coding sequence. The predicted sequence was for a protein containing 688 amino acids, which we have named Macrolin since it was expressed specifically by the giant connective macroglia cells. The calculated molecular mass of Macrolin of 79,004 Da was close to the estimate for the mAb 1A11 antigen of 78 kDa based on SDS-PAGE (Fig. 2). As in the case of Gliarin, Macrolin contains an α-helical rod domain of 354 residues typical of IF proteins (Fig. 3) flanked by a 142 amino acid N-terminal head domain and a 192 residue C-terminal tail

domain. The original cDNA clones identified by mAb 1A11 were also labeled by the Lan3-13 antibody but not by the mAbs 9A8 or G39 (data not shown). These data together with the fact that the labeling of mAb 1A11 and Lan3-13 on immunoblots of leech central nervous system proteins are indistinguishable (Fig. 2) strongly suggest that both mAb 1A11 and Lan3-13 recognize Macrolin.

Filarin. We have previously identified the neuron-specific IF protein Filarin in the leech genus *Haemopis* using the Lan3-8 antibody (Johansen and Johansen, 1995). For comparative purposes we also screened the *Hirudo* cDNA library with the Lan3-8 antibody and identified a full length *Hirudo* cDNA clone of Filarin. *Hirudo* Filarin is a 597 residue IF protein (Fig. 3) with a predicted molecular mas of 67,168 Da. *Hirudo* Filarin is 89% identical to the amino acid sequence of *Haemopis* Filarin.

Phylogenetic analysis. Figure 3 shows a sequence comparison of Gliarin, Macrolin, and Filarin with each other. While the N-terminal amino acid sequences are mainly unique there are many scattered regions of sequence homology in the rod domains and part of the C-terminal tail domains. The most conserved domains between Gliarin, Macrolin, and Filarin as for all IF proteins are at the beginning and end of the rod domain. Macrolin and Gliarin share 49.7% sequence identity in the rod domain whereas Filarin is 36.2% and 46.7% identical in this region to Macrolin and Gliarin, respectively. However, this level of conservation is approximately the same as the amino acid identity to the corresponding sequences in other invertebrate IF proteins. Thus, in order to determine the evolutionary relationship between Macrolin, Gliarin, and Filarin and other members of the IF protein superfamily we constructed phylogenetic trees based on maximum parsimony. Figure 4A shows a consensus tree based on an alignment with all gaps removed (leaving 274 residues) of the rod domains of 36 IF proteins from nuclear, neuronal, and non-neuronal cytoplasmic invertebrate IFs as well as representative sequences from the six classes (I-VI) of vertebrate

IFs. The tree was rooted using sequences from three pseudocoelomate invertebrate IFs (nematode ifA3, ifC1, and cif) as an outgroup. This outgroup was chosen based on the analysis of the topology of individual unrooted maximum parsimony trees. Gliarin, Macrolin, and Filarin were clearly grouped together in a monophyletic clade constituted by neuronal and non-neuronal cytoplasmic coelomate protostomic IFs (Fig. 4A). Interestingly, however, all the nuclear lamins from both vertebrates and invertebrates were grouped together in a monophyletic clade that shared a common ancestor with the monophyletic clade containing the remaining five classes of vertebrate cytoplasmic IFs. Thus, these results suggest that vertebrate cytoplasmic IFs may be more closely related to the nuclear lamins than to the cytoplasmic coelomate protostomic IFs. All the major clades in this analysis have strong bootstrap support of greater than 80% (Fig. 4A, values in italic).

Since the evolutionary relationship between the neuronal and non-neuronal cytoplasmic coelomate protostomic IFs was poorly resolved in the tree depicted in Fig. 4A we realigned these protein sequences with each other in their entirety. After all gaps were removed from this alignment 542 residues remained. Figure 4B shows a rooted consensus tree based on this alignment using the nematode sequence ifA3 as an outgroup; however, the same topology was obtained for unrooted trees. Filarin, Macrolin, and Gliarin are clearly closely related as they formed a monophyletic clade together with an IF sequence from earthworm (Bovenschulte et al., 1995). The earthworm IF is most closely related to Macrolin; unfortunately, its cellular expression has not been determined. Since it formed a clade with Gliarin and Macrolin it would be interesting to know whether it is also expressed by glial cells and might be a functional homolog. Although Filarin is a neuron specific IF, it is clearly not a homolog of the only other neuron-specific IF to be characterized, squid brain IF (Szaro et al., 1991). The three leech IFs were grouped in a monophyletic clade together

with the non-neuronal cytoplasmic IFs separately from a clade formed by the squid brain IF and a homolog recently identified in *Helix* (Adjaye et al., 1995).

DISCUSSION

This study reports the cloning and characterization of two novel IF proteins that are specifically expressed in glial cells of the leech central nervous system. Gliarin is a 71 kDa protein present in all glial cells including both macro- and microglial cells (Lüthi et al., 1994). Using whole-mount and sectioned preparations Lüthi et al. (1994) showed that during development Gliarin is first discernible in 8 day old embryos; however, at this stage only the anlagen for the glial cell bodies are present. The elaborate arborizations of the glial processes progressively develop in the following days until in 21 day old embryos these cells closely resemble those of adult nerve cords (Lüthi et al., 1994). In contrast to Gliarin, Macrolin is expressed in a single glial cell type of which there is only two per segment. These cells are large macroglial cells that envelope the thousands of axons traveling in the two lateral nerves as well as the unpaired Faivre's nerve which form the ganglionic connectives. Thus, these findings imply that the connective macroglial cells express at least two different IF proteins. However, whether Macrolin and Gliarin form independent fibrils of homodimers or whether in these cells they can form heterodimers remains to be determined. The fact that only this type of glial cell exhibits both IFs suggests that their dual expression may be related to the requirement for structural integrity of these cells as they have to maintain extensive arborizations wrapping all axons in the entire connective. While Gliarin and Macrolin are the first glial cell specific IFs to be reported on in invertebrates, the cytoplasmic IF GFAP serves as a marker for astrocytes in vertebrates (Fuchs and Weber, 1994).

All invertebrate cytoplasmic IFs, non-neuronal as well as neuronal, have been found to share a characteristic structure with nuclear lamins consisting of 6 extra heptads in coil 1B as compared to cytoplasmic vertebrate IFs which lack these sequences (Weber et al., 1989; Fuchs and Weber, 1994). This has led to the proposal that all IFs have evolved from a common nuclear lamin-like predecessor (Osborn and Weber, 1986; Dodemont et al., 1990). However, our phylogenetic analysis based on maximum parsimony suggests an alternative hypothesis where the original IF progenitor was a cytoplasmic IF which gave rise to two monophyletic clades, one comprising all coelomate protostomic cytoplasmic IFs and one comprising all nuclear lamins as well as the vertebrate cytoplasmic IFs. In this scenario, vertebrate and perhaps all deutorostomic cytoplasmic IFs may have evolved from a progenitor shared with nuclear lamins by losing the six heptad repeats in coil 1B (Riemer et al., 1998) in an event taking place sometime after the monophyletic clade comprising the coelomate protostomic cytoplasmic IFs had formed. However, it should be pointed out that at the level of the present analysis the ancestral relationship between the major clades could not definitively be resolved. Regardless, an important implication of the topology of the phylogenetic trees is that the presence of neuron and glial cell specific IFs in coelomate protostomes as well as in vertebrates is not of monophyletic origin but rather represents convergent evolution and appears to have arisen independently. This is in contrast to the nuclear lamins from both protostomes and deutorostomes which form a distinct monophyletic clade. This analysis was based on an alignment of the rod domains from which all gaps including that derived from the lack of the 6 coil 1B heptad repeats in vertebrate cytoplasmic IFs was removed eliminating any possible bias on the results of the presence or absence of these sequences in a given IF. Moreover, in all our consensus trees representatives for each of the 6 classes of vertebrate IFs were grouped together in monophyletic clades according to their type supporting the validity of the topology of the

trees. Thus, maximum parsimony analysis based on the amino acid sequence promises to provide a valuable framework for resolving issues of IF gene evolution and how this may relate to the origin of the different intron/exon patterns of the various IF classes (Dodemont et al., 1990).

It has been suggested that the complexity of invertebrate IFs within the nervous system may be less than that seen for vertebrates and that, for example, the splice variants of the squid IFs are an alternative way of generating IF functional diversity (Szaro et al., 1991; Way et al., 1992). This mechanism could function as an adaptation to the relatively smaller genome size of invertebrates as compared to vertebrates where the three major neurofilament components are encoded by distinct genes (Szaro et al., 1991). However, in this paper we show that at least two glial cell specific IFs, Gliarin and Macrolin, are present within an invertebrate nervous system. In addition, the phylogenetic analysis clearly demonstrates that leech Filarin is not a homolog of the squid brain IF indicating that several neuron specific IFs may exist in invertebrates as well. Furthermore, the mAB IFA, which cross-reacts with IF proteins in several invertebrate and vertebrate species (Pruss et al., 1981), recognizes at least 5 prominent protein bands on immunoblots of leech central nervous system proteins (McKay et al., 1984) suggesting that still more IFs await identification. It will be interesting to further investigate the diversity of IFs within the invertebrate nervous system and to determine what role this diversity may play in maintaining the shape and functional integrity of individual neuron and glial cells.

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FIGURE LEGENDS

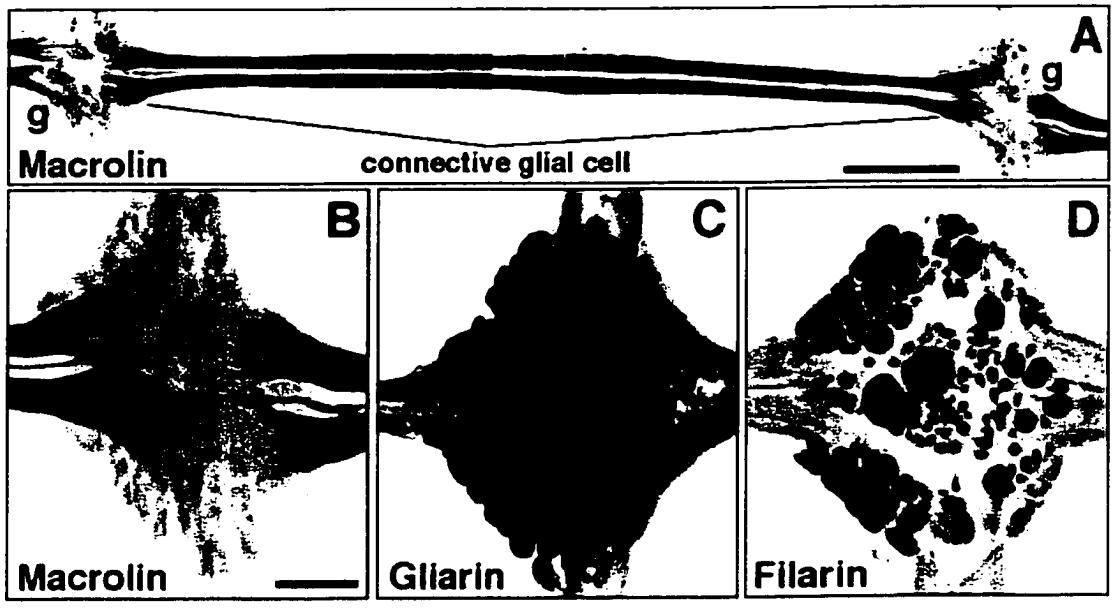
Figure 1 Monoclonal antibody labeling of glial and neuron specific IF proteins in whole mount preparations of leech nerve cords. (A) Labeling of Macrolin in the two giant connective glial cells by mAb 1A11. The micrograph is a composite showing the labeling of the entire connective between two ganglia (g). Scale bar: 600 μm. (B) Higher magnification of one of the ganglia in (A) demonstrating the labeling of Macrolin by mAb 1A11 in the connective macroglial cells at the interphase with the neuropil. (C) Labeling of Gliarin by mAb 9A8 in a leech ganglion. All macro- and microglial cells are labeled including the connective, neuropil, root, and packet glial cells. The wrappings of the packet glial cells of some of the larger neurons are discernible. (D) Labeling of Filarin by the Lan3-8 antibody of all neurons in a leech ganglion. In all figures the ventral side of the nerve cord is shown with anterior to the left. Scale bar for (B), (C), and (D): 150 μm.

Figure 2 Immunoblots of leech central nervous system extracts labeled with the mAbs G39, 9A8, 1A11, Lan3-13, and Lan3-8. Gliarin is identified as a 70 kDa protein by the mAbs G39 and 9A8, Macrolin as a 78 kDa protein by the mAbs 1A11 and Lan3-13, and Filarin as a 63 kDa protein by the Lan3-8 antibody. Molecular weight markers is indicated to the left in kDa.

Figure 3 Alignment and comparison of the predicted amino acid sequences for *Hirudo* Gliarin, Macrolin, and Filarin. Gliarin consists of 639 amino acids with a calculated

molecular mass of 71.3 kDa, Macrolin has 688 residues with a molecular mass of 79.0 kDa, and Filarin is a 597 amino acid protein with a predicted molecular mass of 67.2 kDa. Shared amino acids between these IFs are in white typeface outlined in black and the beginning and end of the coiled-coil rod domain is indicated by arrow heads. Macrolin and Gliarin share 49.7% sequence identity in the rod domain whereas Filarin is 36.2% and 46.7% identical in this region to Macrolin and Gliarin, respectively. These sequence data and the corresponding nucleotide sequences are available from EMBL/GenBank/DDBJ under accession numbers AF101065 (Gliarin), AF101064 (Macrolin), and AF101063 (Filarin).

Figure 4 Consensus maximum parsimony trees derived from alignments of Gliarin, Macrolin, and Filarin with other sequences from the IF superfamily. (A). Consensus tree based on an alignment with all gaps removed (leaving 274 residues) of the rod domains of IF proteins from nuclear, neuronal, and non-neuronal cytoplasmic invertebrate IFs as well as representative sequences from the six classes (I-VI) of vertebrate IFs. The tree is rooted using sequences from three pseudocoelomate invertebrate IFs (nematode ifA3, ifC1, and cif) as an outgroup. (B). Consensus tree based on an alignment with all gaps removed (leaving 542 residues) of the entire sequence of neuronal and non-neuronal cytoplasmic invertebrate IFs. The tree is rooted using the nematode sequence ifA3 as an outgroup. In both (A) and (B) the bootstrap 50% majority rule consensus of 1000 maximum parsimony trees is depicted with associated bootstrap support values. The following IF database sequences were used (corresponding top to bottom to the list in (A)): P22488, S01294, S24545, AF101065, S69003, AF101064, Q01240, X86347, AF101063, S43428, Q03427, P08928, P02546, P02545, P13648, P09010, 998562, S42257, P20152, P0860, P24790, P48675, U59167, P23239, P21807, P03995, P08553, P12036, P35617, P19013, P50446, P05787, P02535, Q99456, P21263, P48681, S46327, AF047657, X070836.


A
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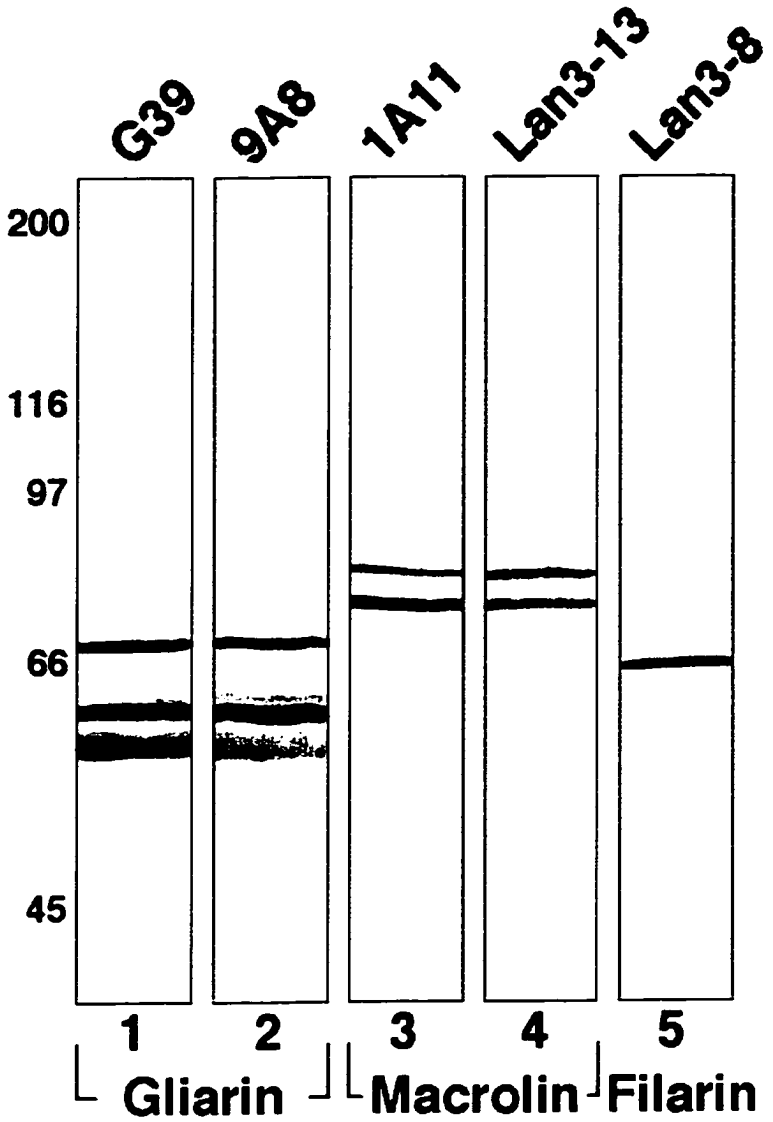
Macrolin
Gliarin
Filarin

Fig. 1


G39
9A8
1A11
Lan3-13
Lan3-8
200
116
97
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1
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Gliarin
Macrolin
Filarin

Fig. 2

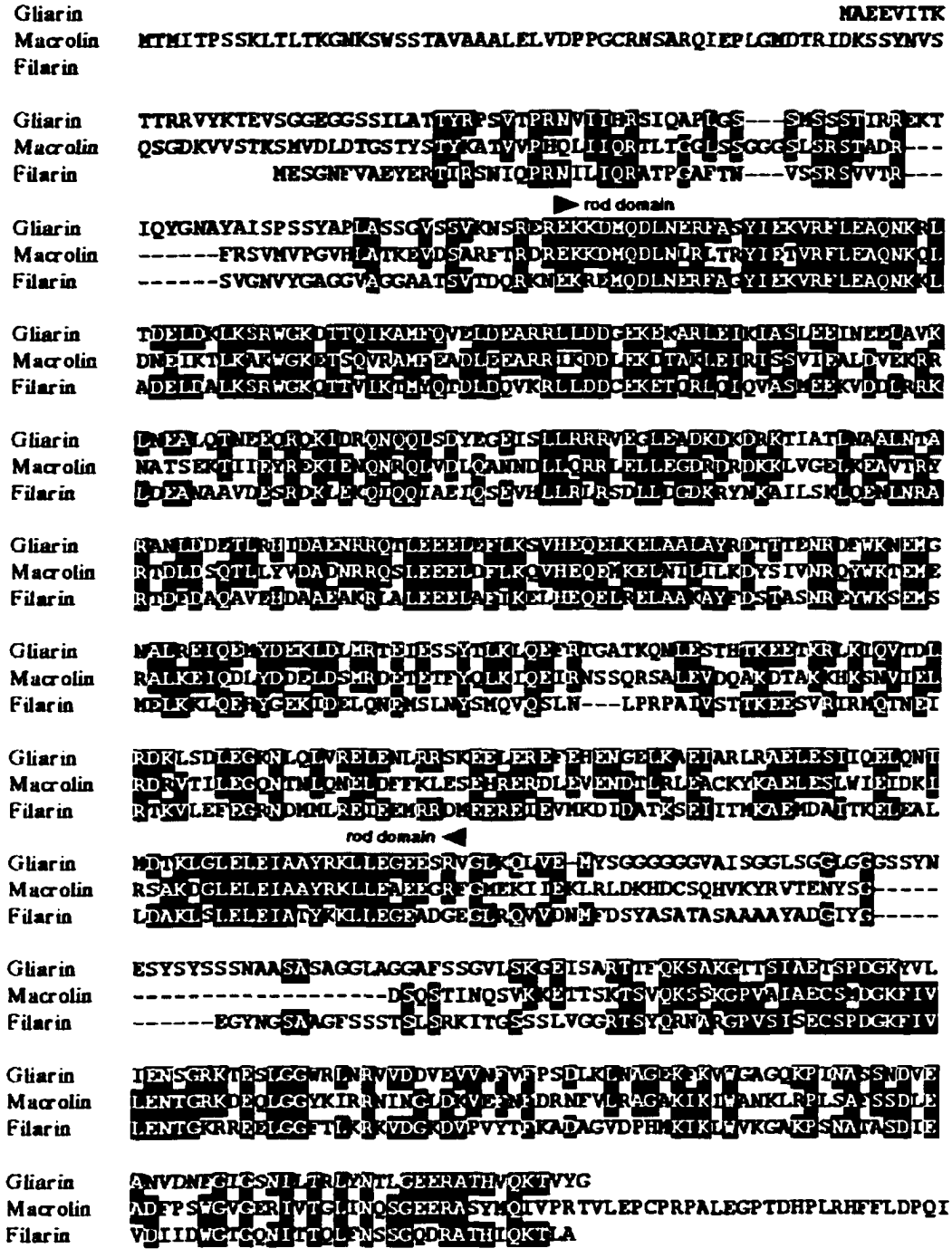

Fig. 3

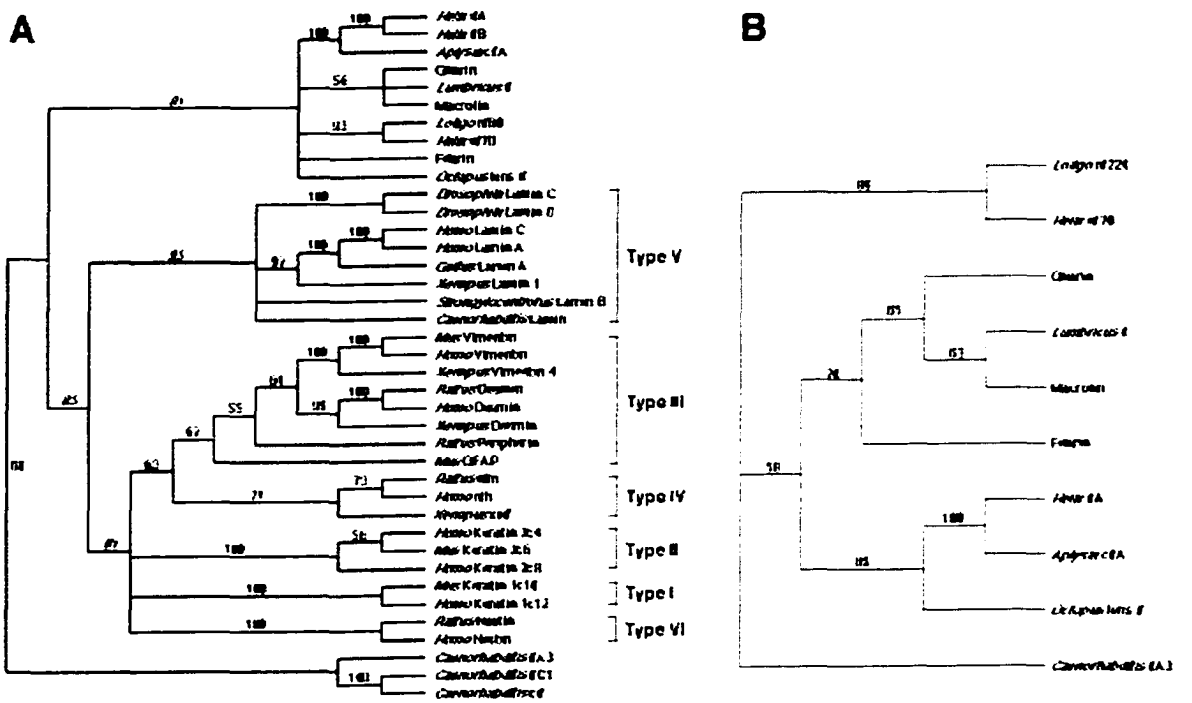

Fig. 4

GENERAL CONCLUSIONS

Tractin, the L1 family member in leech, is highly glycosylated with different glycoepitopes, Lan3-2, Lan4-2 and Laz2-369 (Huang et al., 1997; Jie et al., 2000). Lan3-2 and Laz2-369 glycoepitopes have different oligosaccharide compositions. The antibody labeling of the Lan3-2 glycoepitope is greatly reduced by mannose-BSA, while the labeling of Laz2-369 is greatly reduced by galactose-BSA (Song and Zipser, 1996; Jie et al., 2000). The two glycoepitopes have different temporal and spatial expression patterns in the peripheral sensory neurons and also function differently in neurite outgrowth and synaptogenesis (Song and Zipser, 1995; Tai and Zipser, 1999).

In addition, there are three other glycoepitopes present on the 130 kD protein, Lan2-3, Laz6-212, and Laz7-79 (Peinado et al., 1987, Zipser et al., 1994). Lan2-3 and Laz7-79 glycoepitopes are expressed by small yet distinct subsets of peripheral neurons fasciculating in different axon tracts. Co-immunoprecipitation results with Lan2-3 and Laz7-79 antibody showed that these two glycoepitopes are additional glycomodifications of Tractin. Further functional analysis of these different glycoepitopes would provide more knowledge on how glycoepitopes function in pathfinding, fasciculation and defasciculation in nervous system development.

Tractin is constitutively processed at two cleavage sites (Huang et al., 1997; Jie et al., 2000). One is in the third FNIII domain and the other is proximal to the transmembrane domain. RKRRSR sequences at the first cleavage site conform to the consensus cleavage sequence of furin convertase, RxR/KR (Nakayama, 1997). This cleavage site is found at the same location in the third FNIII domain in most of the L1 CAMs (Hortsch, 1996) including L1, NrCAM, NgCAM, and LAD-1, but not in neuroglian. The site in L1 has been shown to be trypsin (Holm et al., 1995) or plasmin sensitive (Nayeem et al., 1999; Silletti et al., 2000).

When using the furin inhibitor decanoyl-RVKR-chloromethyl ketone (CMK) (Garten et al., 1994; Schäceke et al., 1998), the processing of Tractin is completely blocked, which suggestes that furin directly or indirectly mediates the proteolytic processing. The mutagenesis of RKRRSR to AAAASA totally abolished the processing of Tractin. There is no cleavage product of Tractin when expressed in Lovo cells, a furin deficient human colon cell line (Takahashi et al., 1993; Logeat et al., 1998). These results support the hypothesis that the processing of Tractin at the first cleavage is furin dependent. This furin mediated cleavage might be the general mechanism for the processing of the L1 CAMs.

After cleavage at the third FNIII domain by furin, a fraction of the COOH-terminal fragments are additionally cleaved proximal to the membrane generating a secreted middle fragment (Huang et al., 1997; Jie et al., 2000). We have not yet identified the enzyme responsible in this process. It has been shown that this process in mammalian L1 is mediated by disintegrin metalloprotease ADAM10 (Mechtersheimer et al., 2001). The experiments with the furin site suggested that cleavage at the second site might depend on processing at the first site. There is no cleavage at the second cleavage site when the furin site cleavage is blocked. It also might be that furin is involved in the activation of the enzyme responsible for the cleavage at the site proximal to the membrane.

The cleaved NH_2-terminal fragment is tethered to the cell membrane in S2 cells through its interaction with the COOH-terminal transmembrane fragment. The interaction is mediated by sequences located in the third FNIII domain just distal to the furin cleavage site in the transmembrane fragment. This interaction is necessary for establishing the homophilic cell adhesion in S2 cells (Xu et al., 2003). The interaction domain(s) in the NH_2-terminal fragment responsible for the interaction still need to be further determined. A model of how Tractin might mediate homophilic cell adhesion has been proposed as in figure 1. The

cleaved NH_2-terminal fragment interacts in *cis* with the transmembrane fragment and binds homophilically in *trans* and thus promotes cell-cell adhesion.

In addition to the homophilic interaction, secreted NH_2-terminal fragment might heterophilically interact with other molecules, such as other IgSFs and ECMs. The RGD sequence in the NH_2-terminal fragment might mediate the interaction with certain integrins. The RGD in L1 promotes neurite outgrowth via interaction with $\alpha_v\beta_3$ integrin (Yip et al., 1998). Using ligand screening we might identify other interaction partners of Tractin and provide more information about the heterophilic interaction and its function in neuronal pattern establishment.

Co-immunoprecipitation experiments with leech nerve cord extracts showed some interesting results. Laz6-56 and 1H4 are domain specific antibodies of Tractin, Laz6-56 is specific to the Ig domains and 1H4 is specific to the PG/YG domain. Lan3-2, Laz6-56 and 1H4 antibodies all can co-immunoprecipitate the COOH-terminal transmembrane as well as the cleaved NH_2-terminal secreted fragment, which demonstrates that the two fragments are likely to interact *in vivo*. Lan3-2 antibody immunoprecipitates both the transmembrane 185kD and 165 kD secreted middle fragment, while Laz6-56 antibody immunoprecipitates the transmembrane 185 kD fragment but not the 165 kD secreted middle fragment. These results indicate that in the leech nervous system the NH_2-terminal cleaved fragment only interacts with the transmembrane fragment but not the secreted middle fragment.

Most of the sequence in the COOH-terminal transmembrane 185 kD and 165 kD secreted fragment generated between the two cleavage site is the PG/YG repeats. This region may possess collagen-like structure and properties (Jie et al., 2000). Collagens have been shown to be involved in assembling extracellular matrix (ECM) and activating signal transduction events (Vogel et al., 1997). Collagen XVII is a type II transmembrane protein and epithelial adhesion molecule. Collagen XVII from human skin and epidermal

keratinocytes is posttranslationally cleaved to release a soluble collagen (Schäcke et al., 1998). In addition collagen XIII (Snellman et al., 2000) and ectodysplasin A (Chen et al., 2001) are also constitutively shed from the cell surface to yield a soluble basement membrane collagen. The processing of collage XVII is mediated by ADAM9 and ADAM10, and furin is likely to be involved in the activation of ADAMs enzymes (Schäcke et al., 1998; Franzke et al., 2002). The shedding of collagen XVII ectodomain is associated with altered cell motility (Franzke et al., 2002). The shed ectodomain of XVII contains the Col15 and might exert the function of promoting cell adhesion in a β1 integrin-dependent manner. The shedding may generate a new cell binding site (Tasanen et al., 2000).

After being processed by furin in the third FNIII domain, the COOH-terminal transmembrane fragment of Tractin is further cleaved at a site proximal to the transmembrane domain. The released collagen-like fragment might gain additional interaction sites with other proteins and thus lose its interaction with the NH_2-terminal secreted fragment. Co-immunoprecipitaion results with leech nerve cord support this hypothesis, as the NH_2-terminal fragment only pulls down the 185 kD transmembrane fragment but not the 165 kD secreted fragment. The differences between the secreted and transmembrane fragments containing the PG/YG domain may be a way to fine-regulate signal transduction and/or cell adhesion in axon pathfinding and cell migration. The collagen-like domain is also present in a *Drosophila* cell adhesion molecule, CG6867 (Hynes and Zhao, 2000), which is a type II transmembrane protein with 3 Ig domains and 2 collagen-like domains. Further investigation with both Tractin and CG6867 may reveal a new mechanism of neural cell adhesion molecules in regulating axon outgrowth.

The Lan3-2 recogonized mannoside glycoepitope is presented at the NH_2-terminal fragment of Tractin. Leech nerve cord co-immunoprecipitation results showed that the secreted collagen fragment is not detected in the Laz6-56 ip but is detected in the Lan3-2 co-

ip. This suggestes that the secreted collagen fragment interacts with other protein(s) containing the Lan3-2 glycoepitope. The Lan3-2 glycoepitope is also present on LeechCAM, an ortholog of NCAM in leech (Jie et al., 1999). It has been suggested that the oligo-mannosidic carbohydrates expressed on L1 determine the interaction between L1 and NCAM (Horstkorte et al., 1993). NCAM also interacts with extracellular matrix molecules to mediate neurite outgrowth. Thus LeechCAM might be the interaction partner of the 165 kD secreted fragment, a potential component of extracellular matrix. Antibody perturbation experiments using antibodies against the core proteins of Tractin and LeechCAM showed that not only the nerve pathways are perturbed but the ganglionic development and morphogenesis are affected as well (Johansen and Jellies, unpublished data). Thus Tractin and LeechCAM might function together in mediating the neurite outgrowth and fasciculation in leech nervous system development. Co-ip experiments using the LeechCAM specific antibodies can be carried out to test the hypothesis of the interaction between Tractin and LeechCAM.

A general feature of L1CAM is a diversity of homophilic or heterophilic interaction partners as well as a complex pattern of posttranslational modifications that includes proteolytic processing and glycosylation. Further analysis of the interaction between Tractin and LeechCAM and how different processing fragments of Tractin mediate neurite outgrowth and cell migration using leech neuron cultures would provide a better understanding in the mechanism of L1CAM functions in nervous system development.

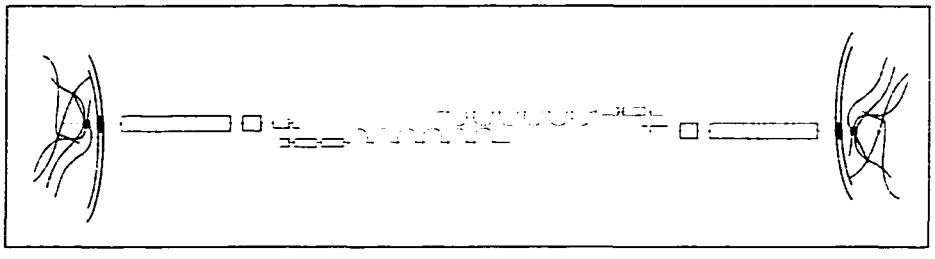

Fig.1 Model for Tractin mediated homophilic adhesion.

This model is proposed based on the results from Tractin mediated S2 cell adhesion. Tractin is processed by furin at the cleavage site in the third FNIIII domain. The NH_2-terminal secreted fragment is tethered to the cell surface through its interaction with the third FNIII domain in the transmembrane fragment. The NH_2-terminal fragments might further homophilically interact in trans to mediate cell-cell adhesion.

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